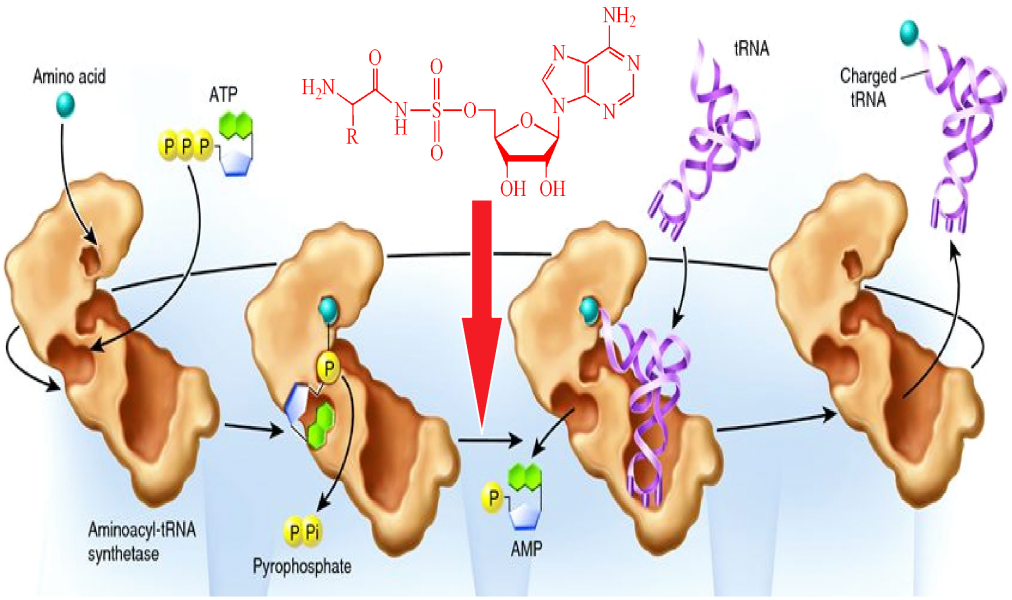
KU Leuven
Biomedical Sciences Group
Faculty of Pharmaceutical Sciences
Rega Institute for Medical Research
Medicinal Chemistry

A Trojan-Horse Strategy for Antimicrobial Therapy



Bharat Gadakh

Doctoral Thesis in Pharmaceutical Sciences
July, 2014
Leuven

KU Leuven
Biomedical Sciences Group
Faculty of Pharmaceutical Sciences
Rega Institute for Medical Research
Medicinal Chemistry

A Trojan-Horse Strategy for Antimicrobial Therapy

Bharat Gadakh

Jury:

Promoter:	Prof. Dr. Arthur Van Aerschot
Chair:	Prof. Dr. Guy Bormans
Secretary:	Dr. Steven De Jonghe
Memmbers:	Prof. Dr. Katrien Lagrou
	Prof. Dr. Augustyns Koen
	Prof. Dr. Serge Van Calenbergh

July, 2014
Leuven

DEDICATED TO MY FAMILY

Acknowledgement

This thesis represents not only my work at the Laboratory of Medicinal Chemistry at Rega Institute for Medical Research; it is also a milestone in my professional career. This thesis is also the result of many experiences I have encountered at KU Leuven from dozens of remarkable individuals who I wish to acknowledge. I find immense pleasure in expressing my sincere gratitude towards all those who have lent a helping hand in the success of my PhD thesis.

First and foremost, I express my deepest sense of gratitude and respect to my venerable promoter ***Prof. Dr. Arthur Van Aerschot*** *for giving me the opportunity to work in the laboratory with outstanding facilities. He has guided my thesis work with noble vision and great wisdom and encouraged me to successfully complete this dissertation. Despite of his busy schedule he was always there to help me with astonishing accuracy. He always supported me throughout my thesis with patience and knowledge while allowing the room to work in my own way. I am fortune to have a promoter like him.*

I would like thank ***Prof. Dr. Mathy Froeyen*** *for molecular modelling studies of the synthesized inhibitors. Without his help, it would not have been possible to explain the biological evaluation results. I would also like to thank* ***Prof. Dr. Eveline Lescrinier*** *for recording and characterization of NMR spectra of few complex molecules and also for scientific discussions pertaining to the 2D NMR assignments. I would like to thank* ***Prof. Dr. Jef Rozenski*** *for hundreds of mass spectra acquisition. I would also like to thank* ***Prof. Dr. Piet Herdewijn*** *for providing healthy work atmosphere with best facilities and motivating environment in the lab.*

I would like to thank my jury members ***Prof. Dr. Guy Bormans***, ***Dr. Steven De Jonghe***, ***Prof. Dr. Katrien Lagrou***, ***Prof. Dr. Augustyns*** *and* ***Prof. Dr. Serge Van Calenbergh*** *for the time they have provided in carefully scrutinising my thesis and coming up with suggestions to improve the quality of the thesis. Additionally I would like to genuinely thank* ***Prof. Dr. Lieve Van Mellaert*** *for her immense support during biological experiments which I have performed in the Laboratory of Molecular Bacteriology (at Rega Institute and also at Gasthuisberg campus). I would like to thank her for her suppor and remarkable suggestions during scientific discussions on antibacterial evaluation and timely help. I would also like to thank people from Laboratory of Molecular Bacteriology: Eveline, Kostas, Nikos, Ans and Sonja De Keyser*

for helping me during my experiments. I would like to thank ***Dr. Stephen Week*** *(Laboratory of Biocrystallography) for IleRS and ITC experiments.*

In my daily work I have been blessed with a friendly and cheerful colleagues: Dr. Gaston Vondenhoff, Milind Saudi, Dr. Omprakash Bande, Dr. Dhuldeo Kachare, Dr. Shriniwas Dumbre, Dr. Puneet Srivastav, Dr. Elizabetta Goraz, Bart Vandenbrande, Dr. Mohitosh Maiti, Dr. Munmun Maiti, Swarup De, Kishore, Dr. Mikhail Abramov, Dr. Hassen Belabed, Dr. Alessandro Stella, Anastasia Parchina, Dr. Anna Ramadori, Dr. Bart Vanderhoydonck, Dr. Johannes Vogel, Dr. Martin Kogler, Dr. Xiaong Song, Dr. Quiya Huang, Dr. Miyeon Jang, Dr. Venkatesham Akkaladevi, Dr. Narsh Badgujar, Dr. Sonja Kovackova, Rania, Elena, Dr. Stafano Agnello, Henri-Philippe Mattelaer, Dr. Marleen Renders, Min Luo, Xiaochen Li, Chao Liu, Xuxiang Chen. Thank you all for making my stay cheerful and memorable.

Special thanks to ***Dr. Gaston Vondenhoff*** *with whom I have worked on several projects and from whom I have learnt to perform in vitro and whole cell assays. I am fortunate to have colleague like him. I would like to thank him for continuous help and constant encouragement.*

Taking this opportunity, I would like to thank ***Luc Baudemprez*** *for NMR acquisition and other technical support. I would also like to thank* ***Guy Schepers*** *and* ***Lia Margamuljana*** *for technical support. I would like thank* ***Sonja Martens*** *for her help in daily work. I would like to thank* ***Chantal Biernaux*** *for all administrative help. I would like to thank* ***Inge Aerts****,* ***Cathy De Meyer*** *and* ***Christane Callebaut*** *for help and support. I should also thank Doctoral School Biomedical Sciences, KU Leuven as well as International Office, KU Leuven for continuous guidance and support throughout my Ph.D. I am indebted to* ***Erasmus Mundus External Cooperation Window (EMECW) Lot 13*** *and* ***KU Leuven*** *for financial support.*

Words do not suffice my feeling for ***Sagnik dada*** *(my elder brother) and mitra* ***Milind*** *who were always with me in good and bad times and keep me motivating to accomplish the task. They taught me the value of friendship, respect for others and understand the feeling of others who care for me. I consider myself extremely fortunate to have friends like them.*

Apart from the professional life, I had a great social life in Leuven. I would like to thank Amrit Paudel, Zola, Dipak Vachhani, Chetan Kulkarni, Chandan Kadur, Vishal Sonje, Hardik Takwani, Janak Thakkar, Mahendra Sonawane, Abhijit Shinde, Parveen Dabas, Parimal Naik, Abhishek Singh, Eshwar Ghumare, Sajin, Niraj Kumar Singh, Dr.

Niraj Mishra, Oliver Gomes, Manish Gupta, Shoaib Iqbal, Ameya and Ashwini Atre, Chandrashekhar Pati Tripathi (CSPT), Shanish, Amol Chaudhary, Rupesh and Rupila Bhabhi. I would like to thank you all for your support and maintaining homely atmosphere which help me keeping focus on my PhD. I am really sorry for those, the names of whom I could not mention because of space limitations. All of you have made this possible, without you I would not have been able to do a PhD. I would like to thank Indian Student Association at Leuven (ISAL) for making me feel at home by organizing trips and other cultural and recreational activities like Indian festivals celebration.

Special thanks to my teachers ***Prof. K.P.R. Kartha****,* ***Mr. S. M. Shelar****,* ***Dr. Parameshwari Halen*** *and* ***Mr. S. V. Amrutkar*** *who inspite of being away, have always been here for me and kept me going. I would also like to genuinely thank* ***Mr. A. M. Shaikh*** *and* ***Mr. A. V. Ugle*** *for help and support throughout my education. These peoples are most precious possessions of my life so far. Many thanks to Tushar Apsunde, Pradeep Jadhavar, Arvind Sonawane, Tushar Sonawane, Rakesh Amrutkar, Hirdesh Sharma, Yogesh Sharma, Srikanta Sahu, RamPrakash Verma and Gopikrishna for moral support.*

I must express my gratitude to my wife ***Sujata****, for her continued moral and emotional support, unconditional love and constant encouragement. I was continually amazed by her willingness to proof read countless pages of my thesis, and by her patience that experienced all of the ups and downs of my research. In final stage of my thesis, I was too busy and couldn't spend quality time with her. I would like to thank her for understanding.*

My parents (Anna and Nani), pappa, mummy, my brothers (Ravindra, Mahendra), brother-in-law Pritam, Sister-in-law Durgeshwari (Mai), Sayali (Didi) and Archana have been greatest source of inspiration and words have no power to express my deep sense of reverence to them. Without their unconditional love, sacrifices and contributions I would not have achieved what I am today. Finally, I would like to thank almighty for giving me strength to complete this task.

Bharat Gadakh

Summary

Over the last seven decades, antibiotics have been essential components of modern medicines and this is one of the leading causes for our increased life expectancy. However, due to development of resistance to the existing antibiotics, there is an urgent need to look for new analogues or new bacterial targets which are essential for the survival of pathogens. Recently, aminoacyl-tRNA synthetases (aaRSs) have emerged as promising and clinically validated targets. Aminoacyl-tRNA synthetases are a family of enzymes which play a key role in the translation process and are responsible for charging tRNA with the correct amino acid. Although these enzymes are conserved during evolution, yet some structural divergence has occurred allowing selective inhibition of the bacterial aaRSs over their human orthologs. Because of their crucial role in protein synthesis and the possibility of selective inhibition, these enzymes have been considered as a prime antibiotic target. Numerous aaRS inhibitors have been reported in the literature, either from natural or synthetic origin. However, none of them (except mupirocin) has been developed yet into a clinically useful antibiotic. Last year, a new drug application (NDA) for Tavaborole (a benzoxaborole derivative) from Anacor Pharmaceuticals has been accepted by the US FDA for the treatment of onychomycosis.

More recently, inhibitors based on the reaction intermediate (aa-AMP) have been designed and evaluated for antibacterial activity. Among them, aminoacyl-sulfamoyl adenosine (aaSA) analogues proved to be the strongest inhibitors of the corresponding aaRSs *in vitro*. However, these analogues could not progress further due to their lack of selectivity and poor cell penetration. In 1998, Cubist Pharmaceuticals reported a series of aryl-tetrazole containing sulfamates as selective aaRS inhibitors. Despite of excellent activity and high selectivity, these analogues could not be pursued further due to their poor cell penetration and high serum albumin binding.

Elaborating on these findings, we attempted to improve the *in vivo* efficacy of the aryl-tetrazole containing sulfamates by coupling them with a siderophore (trihydroxamate or biscatecholate). Although no antibacterial activity was observed in whole-cell assay screening, trihydroxamate-based siderophore-drug conjugates (SDCs) did show nice *in vitro* activity in cell extracts (except ΔABN extracts which lack peptidase activity). Therefore, we concluded that the SDCs are efficiently metabolized by broad-specificity

peptidases to release the active moiety. Thus, failure of uptake is the main reason for the inactivity of these trihydroxamate-based SDCs. Most probably, the iron-transport system may be selective for adenylates or very closely resembling derivatives. In addition, the biscatecholate-based SDC was not metabolized by broad-specificity peptidases as the SDC was protected at its *N*-terminal. Thus failure to release the active moiety is the likely reason for the absence of whole-cell activity of this latter SDC.

Visual inspection of the compounds reported by Cubist Pharmaceuticals, and of albomycin and mupirocin reveals that these compounds vary from aaSA analogues in having either a heterocyclic base or a modified pyrimidine base or no base at all, the latter as found in mupirocin. However, these analogues displayed excellent activity and good selectivity against their respective aaRSs. These observations prompted us to investigate the pharmacophoric importance of the adenine base in aaSA analogues. In chapter 3, different natural and unnatural base containing isoleucyl-sulfamoyl nucleosides were designed, synthesized and evaluated for their antibacterial activity. To our surprise, the order of the *in vitro* inhibitory activity found was uracil (U)> adenine (A) = cytosine (C)> 4-aminobenzimidazole (4-ABI)> 4-nitrobenzimidazole (4-NBI)> guanine (G). Moreover, hexapeptidyl conjugates of these analogues were prepared in an attempt to promote the uptake of these analogues. Unfortunately, only transient antibacterial activity was noticed for purine derivatives (A, I, 4-ABI), while no activity was observed for the *in vitro* strongly active uracil derivative. This observation again supports our hypothesis that the YejABEF transporter is selective for peptidyl-adenylate analogues and closely resembling derivatives.

Part of our synthetic efforts focused on improvement of the *in vivo* efficacy of aaSA analogues by combining them with a peptide carrier (either a siderophore or a McC hexapeptide). During these studies, we consistently observed the formation of a cycloadenosine derivative as a side product. In an effort to reduce this side reaction, we proposed the synthesis of aminoacyl-sulfonamides (aaSoAs) as a potentially more stable alternative for aaSA analogues. Towards this end, we designed and synthesized several aaSoAs. We further compared the activity of these aaSoAs along with the intermediate sulfamate (SA) and sulfonamide (SoA) cores with their corresponding aaSA analogues. It was shown however that these analogues are not able to inhibit the corresponding aaRSs. Only the AspSoA analogue to our surprise showed some selective antibacterial activity

against *E. coli* wt. Although, sulfamate (SA) is not active against the tested aaRSs, it did display a broad-spectrum of activity in whole-cell assays. However, the mode of action of this sulfamate core structure remains to be determined.

Samenvatting

Tijdens de afgelopen zeven decennia maakten antibiotica een essentieel onderdeel uit van ons geneesmiddelenarsenaal en deze vormen één van de belangrijkste redenen voor onze toegenomen levensverwachting. Ten gevolge van de ontwikkeling van resistentie tegen de bestaande antibiotica is er echter een dringende behoefte om op zoek te gaan naar nieuwe analogen of naar nieuwe bacteriële doelwitten die essentieel zijn voor het overleven van pathogene organismen. De laatste jaren werd hiervoor door meerdere onderzoeksgroepen opnieuw sterk gefocust op aminoacyl-tRNA synthetasen (aaRSs) als veelbelovende en klinisch gevalideerde doelwitten. De aminoacyl-tRNA synthetasen zijn een familie van enzymen die een sleutelrol spelen in het vertaalproces en die verantwoordelijk zijn voor het laden van het tRNA met de corresponderende aminozuren. Hoewel deze enzymen over de tijden heen weinig geëvolueerd zijn, is er toch voldoende structurele divergentie opgetreden waardoor selectieve remming van de bacteriële aaRSs mogelijk wordt zonder noemens-waardige inhibitie van hun menselijke analogen. Vanwege hun cruciale rol in eiwitsynthese en de mogelijkheid van selectieve inhibitie, worden deze enzymen dan ook beschouwd als een belangrijk bacterieel doelwit. Talrijke aaRS remmers van natuurlijke of synthetische oorsprong werden reeds gerapporteerd in de literatuur, doch buiten het mupirocine werd echter geen van hen reeds ontwikkeld tot een klinisch bruikbaar antibioticum. Vorig jaar werd wel een nieuwe geneesmiddelen applicatie (New Drug Application, NDA) voor Tavaborole (een benzoxaborool derivaat) van Anacor Pharmaceuticals goedgekeurd door de Amerikaanse FDA voor de behandeling van onychomycose.

De laatste jaren werden meerdere remmers op basis van het reactie tussenproduct van de synthetasen (het aa-AMP) ontworpen en geëvalueerd voor antibacteriële activiteit. Hierbij bleken bij in vitro studies de sulfamaat (aaSA) analogen steeds als sterkste remmers van de overeenkomstige aaRSs naar voor te treden. Toch werd geen vooruitgang meer geboekt met deze analogen, wat te wijten is aan hun gebrek aan selectiviteit en slechte celopname. In 1998 rapporteerde Cubist Pharmaceuticals een reeks van aryl-tetrazool bevattende sulfamaten als selectieve aaRS remmers. Ondanks hun uitstekende activiteit en hoge selectiviteit werden deze analogen niet verder ontwikkeld vanwege hun slechte celpenetratie en hoge serum albumine binding.

Voortbordurend op deze bevindingen hebben we gepoogd om de in vivo werkzaamheid van deze aryl-tetrazool bevattende sulfamaten te verbeteren door hen te koppelen aan een siderofoor op basis van een trihydroxamaat of biscatecholaat structuur. Hoewel er geen antibacteriële activiteit werd waargenomen in cellulaire testen, vertoonden de trihydroxamaat gebaseerde siderofoor conjugaten (SDC's) wel een behoorlijke in vitro activiteit gebuik makend van cel extracten (behalve voor ΔABN extracten waarbij de peptidase activiteit ontbreekt). We concluderen bijgevolg dat de SDC's efficiënt worden gemetaboliseerd door brede specificiteit peptidasen waarbij het actieve deel vrijgezet wordt. Gebrekkige opname is bijgevolg de belangrijkste reden voor de inactiviteit van deze trihydroxamaat gebaseerde SDC's. Waarschijnlijk is het ijzertransportsysteem hierbij selectief voor adenylaat structuren of sterk gelijkende derivaten. Bovendien werd het biscatecholaat gebaseerde SDC niet gemetaboliseerd door brede specificiteit peptidasen wanneer dit conjugaat beschermd was aan het *N*-terminaal uiteinde. Het niet vrijkomen van het actieve deel is bijgevolg de meest plausibele reden voor de afwezigheid van activiteit in celculturen.

Uit visuele inspectie van zowel de Cubist Pharmaceuticals verbindingen als van albomycin en mupirocine, blijkt dat deze verbindingen verschillen van de aaSA analogen wat betreft het base gedeelte, met ofwel een ongewone heterocyclische verbinding, een gemodificeerde pyrimidine ring of afwezigheid van een base zoals bij het mupirocine. Nochtans vertonen deze analogen een uitstekende activiteit en een goede selectiviteit tegen hun respectieve aaRS doelwit. Deze waarnemingen vormden voor ons de aanleiding om het belang van de adenine base in aaSA analogen te onderzoeken. In hoofdstuk 3 werden verschillende isoleucyl-sulfamoyl nucleosiden met een natuurlijke of onnatuurlijke base geconcipieerd, gesynthetiseerd en geëvalueerd op hun antibacteriële werking. Tot onze verbazing bleek hierbij de volgorde van de in vitro remmende activiteit als volgt: uracil (U)> adenine (A) = cytosine (C) > 4-aminobenzimidazole (4-ABI) > 4-nitrobenzimidazole (4-NBI) > guanine (G). Tevens werden hexapeptidyl conjugaten van deze analogen bereid in een poging om hun opname te promoten. Helaas werd slechts een voorbijgaande antibacteriële activiteit bemerkt voor de purinederivaten (A, I, 4-ABI), terwijl geen activiteit werd waargenomen voor het in vitro sterk actieve uracil derivaat. Deze waarneming ondersteunt opnieuw onze hypothese dat de YejABEF transporter selectief peptidyl-adenylaat analogen of sterk gelijkende derivaten herkent.

Een deel van onze synthetische inspanningen was gericht op verbetering van de in vivo werkzaamheid van aaSA analogen door verbeterde opname, wat kon beoogd worden door ze te conjugeren met een peptide (zij het een siderofoor of een McC signaalpeptide). Tijdens onze studies hebben we hierbij steeds de vorming van een cycloadenosine derivaat waargenomen als bijproduct. In een poging deze nevenreactie te beperken beoogden we vervolgens de synthese van aminoacyl-sulfonamiden (aaSoAs) als een potentieel stabieler alternatief voor aaSA analogen. Hiertoe werden verschillende aaSoAs ontworpen en gesynthetiseerd. We vergeleken hierbij de biologische activiteit van deze aaSoAs alsook deze van de intermediaire sulfamaat (SA) en sulfonamide (SoA) kernen met deze voor de overeenkomstige aaSA analogen. Hierbij werd jammer genoeg aangetoond dat deze sulfonamide analogen niet in staat zijn de overeenkomstige aaRSs te remmen. Alleen het AspSoA analoog bleek enige selectieve antibacteriële activiteit te vertonen tegen wild type *E. coli*. Hoewel het sulfamaat (SA) in vitro niet actief bleek tegen de geteste synthetasen, vertoont dit product wel een breed spectrum van activiteit in cellulaire assays. Het werkingsmechanisme hiervan dient echter nog te worden bepaald.

List of abbreviations

A	adenine
aa	amino acid
aaRS	aminoacyl-tRNA synthetase
aaRSi	aminoacyl-tRNA synthetase inhibitor
aaSA	5'-*O*-(*N*-aminoacyl)-sulfamoyladenosine
aaSoA	5'-(*N*-aminoacyl)-sulfonamido-5'deoxyadenosine
aa-tRNA	aminoacyl-tRNA
ABC	ATP binding cassette
4-ABI	4-Amino-benzimidazole (1,3-dideazaadenine)
ΔABN	Bacterial strains lacking peptidase A, B and N (pepABN)
ACN	Acetonitrile
ADP	Adenosine 5'-diphosphate
AdT	aminoacyl-tRNA amidotransferase
AHO	N^{δ}-acetyl-N^{δ}-hydroxy-L-ornithine
AMP	adenosine-5'-monophosphate
AspSoA	5'-(*N*-aspartyl)-sulfonamido-5'deoxyadenosine
ATCC	American type cell culture collection
ATP	Adenosine-5'-triphosphate
Boc	*tert*-butoxycarbonyl
C	cytosine
Cbz	Benzyloxycarbonyl
$CDCl_3$	Deuterated chloroform
*m*CPBA	*m*-Chloroperoxybenzoic acid
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	Dicyclohexylcarbodiimide
DCDMH	1,3-Dichloro-5,5-dimethyl hydantoin
DCM	dichloromethane
DEAD	diethyl azodicarboxylate
2,3-DHB	2,3-dihydroxybenzoic acid
DIAD	diisopropyl azodicarboxylate
DIC	diisopropylcarbodiimide

DIPEA	*N,N*-diisopropylethylamine
DMAc	*N,N*-dimethylacetamide
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DTD	D-aminoacyl-tRNA deacylase
DTT	1,4-dithiothreitol
EDCI·HCl	*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
EF	elongation factor
FDA	food and drug administration
Fmoc	9H-fluoren-9-ylmethoxycarbonyl
G	guanine
GluAdT	Glu-tRNA^{Gln} amidotransferase
HOBt	*N*-hydroxybenzotriazole
HBTU	*O*-Benzotriazole-*N*,*N*,*N'*,*N'*-tetramethyl-uronium-hexafluoro-phosphate
HPLC	high performance liquid chromatography
HRMS	High resolution mass spectrometry
HTS	High throughput screening
IC_{50}	half maximal inhibitory concentration
LB	Lysogeny Broth
LPS	lipopolysaccharides
McC/MccC	Microcin C
MHA	Muller-Hinton agar
MIC	minimum inhibitory concentration
mM	millimolar
µM	micromolar
mRNA	messenger ribonucleic acid
MS	mass spectrometry
4-NBI	4-nitro-benzimidazole
nM	nanomolar
NMR	nuclear magnetic resonance

NRPS	non ribosomal peptide synthetase
-OSu	*N*-hydroxysuccinimide ester
PBP	penicillin binding protein
PDF	peptide deformylase
PIDA	Phenyliodinediacetate
PP	pyrophosphate
PTSA	*p*-toluenesulfonic acid
rt	room temperature
SA	5'-sulfamoyl adenosine (or sulfamate)
SDC	siderophore-drug conjugate
SoA	5'-sulfonamido-5'-deoxyadenosine
*t*Bu	*tert*-butyl
TBDMS	*tert*-butyldimethylsilyl
TEA	Triethylamine
TEAB	triethylammonium bicarbonate
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
THF	tetrahydrofuran
tRNA	transfer ribonucleic acid
wt	wild type
Z	benzyloxycarbonyl

IUPAC abbreviations for amino acids

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cystein	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Contents

1 General Introduction

1.1 Introduction

Alarming resistance to the existing antibiotics provides continuous stimulus to search for new antimicrobial agents or new cellular targets which are essential for the survival of the pathogen. In recent years, aminoacyl-tRNA synthetases (aaRSs) have emerged as an essential and clinically validated target for the development of antimicrobial agents. Given their indispensible role in protein synthesis and significant divergence between prokaryotic and eukaryotic aaRSs, these enzymes are considered as a promising target for the development of antimicrobial agents. This chapter covers an overview on the role of aaRSs in protein synthesis, and their classification, and discusses the existing aaRS inhibitors from natural and synthetic origin and recent developments in the field. This is followed by a brief discussion on the poor *in vivo* efficacy problem associated with the *in vitro* strongly inhibitory aminoacyl-sulfamoyl adenosines (aaSAs) and a Trojan-horse strategy to overcome it. Being aaRS inhibitors and Trojan-horse antibiotics, microcin C and albomycin will be discussed in detail as they form basis for this thesis.

Human evolution can be viewed from a medical point of view as a continuous fight between human and its pathogens.[1] Until the first half of the twentieth century, infectious diseases were the leading cause of mortality and morbidity. Therefore it is not surprising that earlier research was focused on infectious diseases with the main concern on prevention or therapy of infectious diseases. There are several landmark discoveries during the course of the last century such as the discovery of penicillin by Alexander Fleming in 1929 and the first introduction of the sulpha drugs by Domagk in 1932. There also was a tremendous increase in new antimicrobials during 1940-1960. Till early 1970s, mankind was confident that infectious diseases can be prevented and controlled with the existing antimicrobial agents. In 1969, Surgeon General William H. Stewart told the United State congress that it was time to 'close the book on infectious diseases'.[2] With victory declared, efforts for research on infectious diseases were reduced or almost eliminated. However, infectious diseases are still the second leading cause of mortality worldwide, causing over 13.3 billion deaths per year.[3] This is the result of the emergence

of new diseases, the re-emergence of diseases once controlled and more specifically the development of antimicrobial resistance.

Antibiotic resistance can be divided in two types, the natural or intrinsic and the acquired resistance. Acquired resistance is the major mechanism.[4] Herein, bacteria acquired resistance through three major mechanisms: (1) prevention of accumulation of the antimicrobial agent either by decreasing influx or increasing efflux (2) mutation(s) leading to alteration in the drug target which therefore prevent drug binding or effective action (3) Antibiotic inactivation either by hydrolysis or modification.[5] In contrast, intrinsic resistance is the result of general adaptive processes and is not necessarily linked to any specific class of antimicrobials. An example includes the absence of an uptake transport system for antimicrobials or poor permeability of the outer membrane of Gram-negative bacteria. The cell envelop permeability barrier is particularly strong in Gram-negative pathogens like *Pseudomonas aeruginosa* and other Gram-negative bacteria. *P. aeruginosa* is well known for its intrinsic resistance due to its rigid, less diffusible cell membrane and its ability to develop multidrug resistance following therapy.[5a] Many antimicrobial agents are inactive against certain strains of bacteria because they simply can not enter the cell.[6]

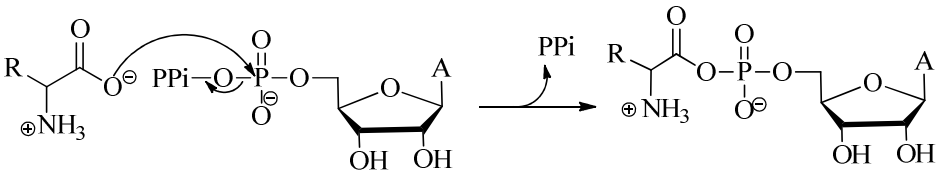
The bacterial resistance to every antimicrobial agent is unavoidable and provides continuous stimulus to search for new bacterial targets which are essential for the survival of microbes or for new antimicrobial agents which preferably act by novel mode(s) of action. Strategies to overcome resistance involve further development of the existing classes of antibiotics and the use of combinations of existing antibiotics, as well as searching for new classes of antibiotics. The former strategy is likely the most promising as it is cost effective and yields new antibiotics in a relatively short period. However, there is always a greater risk of rapid reoccurrence of resistance. Therefore efforts should be directed to the development of new classes of antibiotics with preferably different mode(s) of action to prevent development of cross-resistance. In theory, a new class of antibiotics must fulfil three criteria in which the new target must be essential for the bacterial cell function (i.e. the target should be validated), the new antibiotic must be very selective for the bacterial target and bacteria should not be able to develop resistance to these class of antibiotics.[7] Alternatively, efforts should be invested to look for unexploited and essential cellular targets. One such target is aminoacyl-tRNA synthetase.

1.2 Aminoacyl-tRNA synthetases (aaRSs)

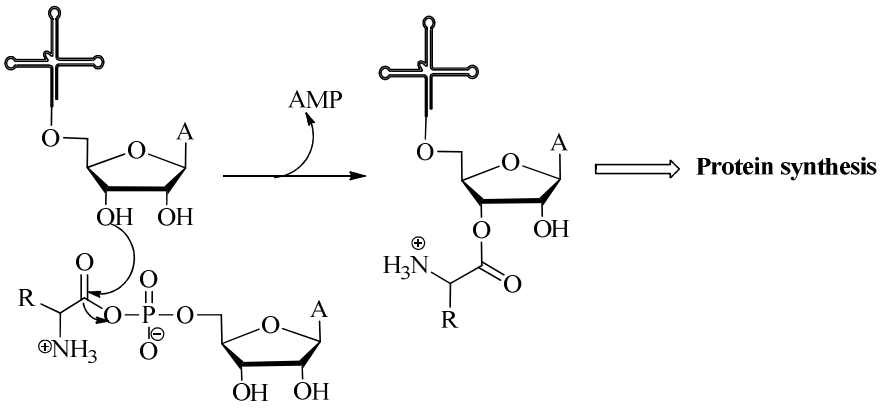
In last couple of decades, aminoacyl-tRNA synthetases (aaRSs) have emerged as a promising and clinically validated target for the development of new antimicrobial agents as exemplified by the clinical use of mupirocin (Bactroban® by GSK).[8] Aminoacyl-tRNA synthetases (aaRSs) are at the heart of protein synthesis and play an indispensible role in translating the genetic code. They are present in all living organisms and are responsible for ligation of the correct amino acid to their cognate tRNAs which are subsequently used in protein synthesis.[9] The fidelity and accuracy of the translation is largely dependent on the ability of each of tRNA synthetases to ligate the correct amino acid to their correct tRNA because the nature of interaction between tRNA and mRNA is independent of the nature of the amino acid attached to tRNA.[9b] As a general rule, there is one tRNA synthetase for each of the 20 amino acids, but there are some exceptions to this rule, of which the most notable is glutaminyl-tRNA synthetase being absent in Gram-positive bacteria. Instead, these bacteria have an amidotransferase which converts mischarged Glu-tRNA^{Gln} to Gln-tRNA^{Gln}.[10]

1.2.1 Role of aminoacyl-tRNA synthetase in protein synthesis

The aminoacylation of tRNA occurs in a two steps process (Figure 1-1).[11] In the first step, nucleophilic attack of the α-carboxyl of the amino acid takes place on the α-phosphate of the adenine triphosphate (ATP) to form an enzyme bound mixed anhydride (i.e. aminoacyl adenylate, aa-AMP) with the displacement of pyrophosphate (PPi). In the second step, the activated amino acid is transferred to the 3'-terminal adenosine of the corresponding tRNA to form aminoacyl-tRNA (aa-tRNA) and adenosine monophosphate (AMP). This esterification step involves a nucleophilic attack by either the 2'- or 3'-ribose hydroxyl group of the terminal adenosine of the tRNA generating the activated aa-tRNA. These correct aa-tRNAs interact with elongation factor (EF-1a in eukaryotes and Archea, EF-Tu in prokaryotes) to translate mRNA within the A site of the ribosome.[12]



a) Formation of enzyme bound aa-AMP complex.



b) Transfer of amino acid to the tRNA

Figure 1-1: Aminoacylation of tRNA catalyzed by aaRS. The aminoacylation reaction depicted here is catalyzed by a class II aaRS (aminoacylation of the 3'-hydroxyl of terminal adenosine). In case of class I aaRS, the 2'-hydroxyl of the terminal adenosine is acylated.

1.2.2 Aminoacyl-tRNA synthetase: an attractive target

The aaRSs represent an ideal and validated target for the discovery of new antimicrobials for a number of reasons. Firstly, these enzymes play a crucial role in translating the genetic code and thus are of vital importance for the survival of the pathogen.[13] Secondly, although the function of these enzymes is conserved throughout evolution, yet significant structural differences have occurred between prokaryotes and eukaryotes. Therefore selective inhibition of the bacterial aaRS over its human orthologs can be achieved.[14] Thirdly, significant homology exists among prokaryotes implying that aaRS inhibitors may yield broad-spectrum antibiotics. Furthermore, these enzymes are stable, soluble, easy to purify and can be assayed by high throughput screening (HTS).[15] Development of the assay method is always the main bottle-neck for the discovery of new antibacterials. Because of their conserved functionality, the assay developed for one aaRS can be easily adapted for other aaRS.[16] Crystal structures of many aaRSs are now

available which further helps in rational drug design and thus accelerates the process of antibiotic development.[17]

1.2.3 Classification of aminoacyl-tRNA synthetases

Despite of the conserved mechanism of catalysis of all 21 aaRS, they are classified into two unrelated classes: 11 in class I and 10 in class II (with LysRS found in both classes). This classification is mainly based on their structural differences. The active site of class I synthetases consists of two dinucleotide-binding Rossmann folds with HIGH and KMSKS motif which stabilizes the transition state.[18] On the contrary, the active site of class II synthetase contains a barrel like structure of anti-parallel β-sheets surrounded by loops and α- helices that provides a rigid template for the recognition and binding of the amino acid and ATP.[15-19] In addition, a class I synthetase binds ATP in the extended conformation whereas a class II synthetase binds the nucleotide in the bent conformation.

Table 1-1: Summary of structural differences and classification of tRNA synthetases (Copied from ref.[19a])

Feature	**Class I tRNA synthetase**			**Class II tRNA synthetase**		
Active site architecture	Two dinucleotide-binding Rossmann folds			Barrel like anti-parallel β-sheets surrounded by α-helices		
Site of esterification	2'-hydroxyl			3'-hydroxyl		
Enzyme approaches tRNA	From minor groove			From major groove		
Sub-classification	**Ia**	**Ib**	**Ic**	**IIa**	**IIb**	**IIc**
	LeuRS			HisRS		
	IleRS	GlnRS	TyrRS	ProRS	AsnRS	
	ValRS	GluRS	TrpRS	SerRS	AspRS	PheRS
	CysRS	LysRS-I		ThrRS	LysRS-II	
	MetRS			GlyRS		
	ArgRS			AlaRS		

Furthermore, there is a difference in the position of esterification at the ribose of the 3'-adenosine of the tRNA with amino acid. Class I synthetases esterify at the 2'-hydroxyl whereas class II synthetases esterify at the 3'-hydroxyl of the terminal ribose.[20]

This can be explained by the binding of the tRNA to its synthetase. Class I enzymes approach the acceptor stem of the tRNA from the minor groove side with the variable loop facing the solvent, whereas class II synthetases approach the major groove side of the acceptor stem and the variable loop faces the synthetase. However this generalization of modes of tRNA binding being specific for each class of aaRS remains somewhat unclear due to the fact that very few crystal structures of aaRSs contain tRNA. Moreover, extensive biochemical studies revealed that AlaRS recognizes the minor groove of the $tRNA^{Ala}$ although it belongs to class II of the synthetases.[21] However, the recognition of the cognate tRNA by each aaRS involves the discriminator base N73, the acceptor stem and the anti-codon present in the tRNA. To maintain accuracy of aminoacylation with an error rate of ~1 in 10,000 all aaRS contain a distinct structural domain for anti-codon recognition. Some aaRS contain a zinc-binding domain for the acceptor stem recognition. A series of intricate contacts with the substrate amino acid and the cognate tRNA with stabilization of the transition state provide the first level of specificity.[19b] However certain amino acids (e.g. Val and Ile) are structurally so similar that misactivation of the amino acid may occur. Therefore two different proof reading mechanisms exist to decrease the error rate. These mechanisms have been discussed in the next section. Based on their sequence homology and domain architecture, tRNA synthetases are further classified into subgroups has been summarized in Table 1-1.

1.2.4 Editing mechanism of aaRS

As mentioned earlier, the accuracy of protein synthesis not only depends on the recognition of the cognate tRNA and the correct amino acid by aaRS but also on subsequent editing of errors.[22] Since the tRNAs are relatively large molecules with adequate structural differences, their selection is not a big problem.[23] Moreover the accuracy is further enhanced by stabilization of the transition state for aminoacylation in cognate tRNA bound aaRS complexes.[24] In contrast, amino acids are small molecules and differ from each other only by their side chain. The structural differences between the amino acids are very marginal so that aaRS fail to distinguish between them with adequate selectivity leading to misactivation of an amino acid. For example, the IleRS may misactivate valine with an error rate of 1 in 150 whereas the error rate in misinterpretation of the isoleucine codon as valine is only 1 in 3000. As shown in Figure 1-2, the aaRS is able to minimize errors in this selection at two points in the

aminoacylation reaction. At a first point as an aaRS bound aa-AMP (pre-transfer editing) and at the second point as an enzyme bound aminoacyl-tRNA (post-transfer editing). In pre-transfer editing, the misactivated amino acid (xx-AMP, Figure 1-2) is hydrolysed back into the amino acid and AMP, whereas in the post-transfer editing, the incorrectly aminoacylated tRNA (xx-tRNA, Figure 1-2) is hydrolysed into the amino acid and the tRNA.

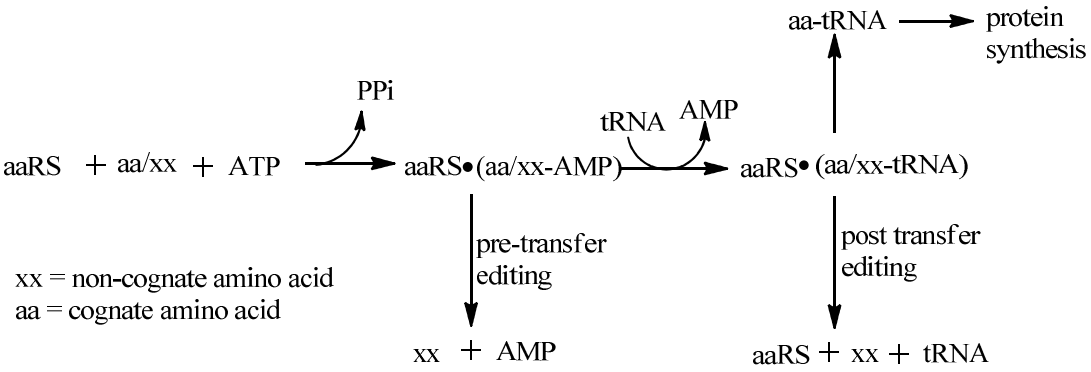


Figure 1-2: Editing pathways of aminoacyl-tRNA synthetases

The catalytic site of aaRS is specific enough to activate and transfer only the correct amino acid. This accuracy can be achieved by the recognition of specific properties of the cognate amino acid and steric exclusion of the amino acids with bulkier side chains. In addition, the difference in the sugar puckering and the orientation of the C-4'-C-5' of the ribose play an important role to discriminate between the cognate and non-cognate aa-AMP.[25] Therefore, the first sieve serves to exclude the amino acids bulkier than the cognate amino acids. However, the amino acids with smaller size which can form sufficient interaction with the aaRS may pass through the first sieve and may be incorrectly activated. Therefore, those aaRS where amino acid discrimination is threatened have evolved with an editing site to meet the fidelity requirement of the cell. The editing site serves as a second sieve and acts by hydrolysing the non-cognate aa-AMP. The editing site is too small to fit the cognate amino acid, therefore capable of hydrolysing non-cognate amino acid (with smaller size). This is called the double sieve model as proposed by Fersht A. R. *et al.*[26] The existence of pre-transfer editing was first proposed by Berg and co-workers. However, because of the transient nature of the intermediate aa-AMP, it has been long controversial to explain the accuracy and fidelity of aminoacylation.[22, 27] It was then inferred from the early pre-steady state kinetic experiments on IleRS by Fersht *et al.* where accumulation of the non-cognate Val-

$tRNA^{Ile}$ was much lower than expected. This study suggested pre-transfer editing of the misactivated valine (Val-AMP) by hydrolysis of Val-AMP.[26] This mechanism of editing by aaRS (pre- or post-transfer editing) has been review by Martinis and Boniecki.[28]

1.2.5 Indirect biosynthesis: relaxed specificity in AspRS and GluRS

Although the notion that all living organisms have one aaRS for each amino acid is widespread, there are a few exceptions like archea and some of the bacteria. For example GlnRS is absent in some *Bacilli* where as GlnRS and AsnRS are absent in archaebacteria.[10a, 29] They have indirect pathway for synthesis of Gln-$tRNA^{Gln}$ and Asn-$tRNA^{Asn}$, both proceeding via mis-aminoacylated intermediates Glu-$tRNA^{Gln}$ and Asp-$tRNA^{Asn}$ respectively. However, these mischarged tRNA can not participate in protein synthesis as they are not recognized by the elongation factors and therefore the accuracy and fidelity of the translation is maintained.[9c, 30] The basis for this relaxed specificity of GluRS and AspRS is found to be the lack of recognition of the third position of the anticodon in tRNA. For example, AspRS can recognize both $tRNA^{Asp}$ (GUC anticodon) and $tRNA^{Asn}$ (GUU anticodon).[30c] The same is also true for GluRS.[30b] The mischarged tRNAs are then converted to their correctly charged tRNA by a tRNA dependent amidotransferase (AdT). Two types of amidotransferase have been found in bacteria and archea. The GatCAB is a heterotrimeric AdT and is found in most of the bacteria and some archea and can use Glu-$tRNA^{Gln}$ and Asp-$tRNA^{Asn}$ as substrates,[31] whereas GatDE is a dimeric AdT and is found only in archea and uses only Glu-$tRNA^{Gln}$ as a substrate.[32] The transamidation reaction requires an acceptable amide donor (such as glutamine, asparagine or ammonia), the mischarged tRNA, ATP and Mg^{2+}. The transamidation reaction may proceed via an aminoacyl phosphate intermediate (e.g. **1.6**, Figure 1-4) as it has been observed that ATP is cleaved to ADP when incubated with Glu-$tRNA^{Gln}$. The role of the different subunits of GatCAB and GatDE is not yet clear. The transamidation of Asp-$tRNA^{Asn}$ and Glu-$tRNA^{Gln}$ has been reviewed elsewhere.[10a, 16] Direct and indirect synthesis of Gln-$tRNA^{Gln}$ is depicted in Figure 1-3 as an example. A similar pathway is also followed for synthesis of Asn-$tRNA^{Asn}$.

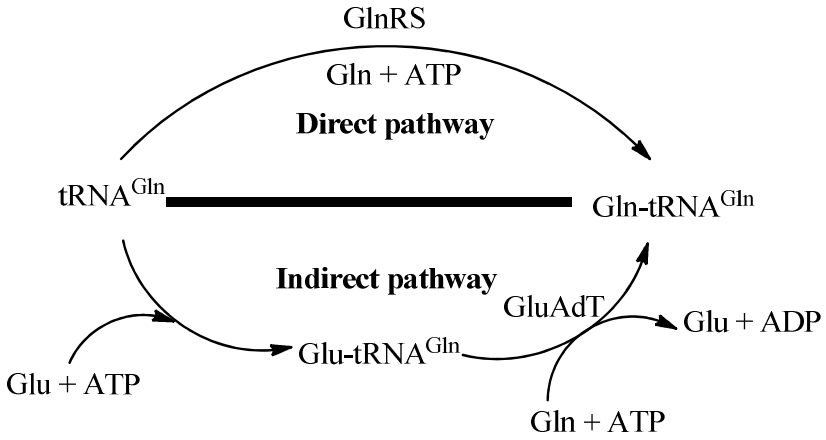


Figure 1-3: Dual pathways for synthesis of Gln-tRNA^{Gln}. The upper half depicts the direct pathway catalyzed by GlnRS whereas the lower half depicts the indirect pathway and is catalyzed by non-discriminating GluRS followed by transamidation by GluAdT. (Figure adapted from the ref.[31a])

As the transamidation pathway is not present in eukaryotic cells, amidotransferases (AdTs) are interesting targets for the development of antibacterial agents. Inhibition of amidotransferases (AdTs) should give the same effect as aaRS inhibition. Hereto, some analogues resembling either the 3'- end of the aminoacyl-tRNA (**1.1** and **1.2**, Figure 1-4) or transamidation reaction intermediates (**1.6**) have been synthesized and evaluated for their antibacterial activity.[31b] However, it has been found that aspartycin (**1.3**, Figure 1-4) is not a substrate for AdT, whereas glutamycin (**1.4**, Figure 1-4) strongly inhibits AdT (GatCAB) with K_i of 0.105 mM. The Asp-KP (**1.7**) failed to inhibit AdT probably due to lack of the adenosyl moiety. Moreover, a series of chloramphenicol analogues was synthesized and evaluated as inhibitors of *H. pylori* GatCAB transamidase. The compound **1.7** proved to be the most active analogue of this series with respect to Asp-tRNA^{Asn} with a K_m of 2 μM and K_i value of 27 μM.[33]

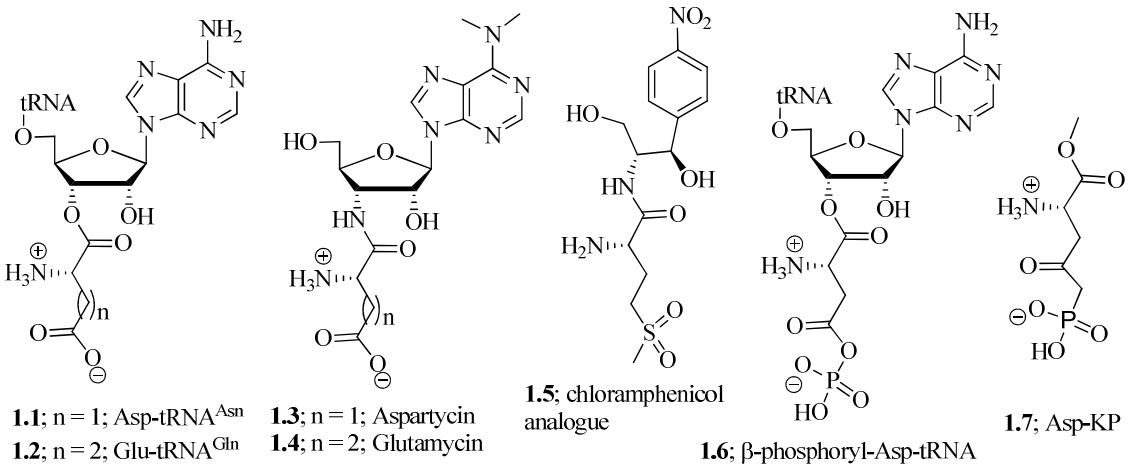


Figure 1-4: Structures of the aminoacyl-tRNA substrates (**1.1** and **1.2**) and respective inhibitors (**1.3**, **1.4** and **1.5**) along with the intermediate in the amidotransferase reaction (**1.6**) and its inhibitor (**1.7**) respectively.

1.3 Existing tRNA synthetase inhibitors

To date, numerous aaRS inhibitors have been reported in the literature either from natural or synthetic origin. Most of them are competitive inhibitors and act by occupying the active site of the corresponding aaRS. Recently, aaRS inhibitors which selectively target the editing site have been reported. Some of the most important aaRS inhibitors from natural and synthetic origin will be discussed in the next section.

1.3.1 Natural products as tRNA synthetase inhibitons

Natural products illustrate a diverse set of chemical structures as aaRS inhibitors of which most important natural products are depicted in Figure 1-5. Although the natural products demonstrate the possibility of selective inhibition, yet none of these analogues (except mupirocin) reached the clinic.[16] Most important natural products as aaRS inhibitors have been described in following section.

Mupirocin: only aaRS inhibitor on the market

Mupirocin (pseudomonic acid, **1.8**, Figure 1-5) is a natural antibiotic produced by *Pseudomona fluorescens* and has a unique chemical structure. It consists of a short fatty acid (9-hydroxy nonanoic acid) linked to monic acid through an ester linkage with the tail portion closely resembling the isoleucyl moiety.[20a, 34] It acts as a competitive IleRS inhibitor and binds to IleRS in a very similar way as Ile-AMP. The tetrahydropyran ring

takes the place of the ribose, with the epoxide ring occupying the same domain as the phosphate group, the tail portion mimicking the isoleucine and the fatty acid chain binding at the adenine binding pocket. Mupirocin is mainly active against most of the Gram-positive bacteria, including *S. aureus* (MIC = 0.06-0.5 μg/mL) and *S. pyogens* (MIC = 0.12-0.5 μg/mL). It is barely active against Gram-negative bacteria (MIC 128 μg/mL for *E. coli*).[34a, 35] It is the only aaRS inhibitor used clinically and marketed as Bactroban® by GSK. Because of the poor bioavailability, and instability owing to its ester function, mupirocin is used as a topical ointment.[36] Although mupirocin is less active against Gram-negative bacteria, high local concentration can be achieved by topical use.

Unfortunately, the clinical use of mupirocin is further restricted due to the emergence of high levels of resistance encoded by the plasmid mediated *mupA* gene.[37] The *mupA* gene codes for the structurally different IleRS which is not inhibited by mupirocin.[38] Because of limited systemic stability of mupirocin, there is a long standing interest in developing mupirocin-based analogues with improved pharmacological properties such as hydrolytic stability, improved potency and selectivity.[39] These analogues have been discussed under the mupirocin-based synthetic aaRS inhibitors section.

Indolmycin

Indolmycin (**1.9**, Figure 1-5) is a secondary metabolite produced by *Streptomyces griseus* ATCC 12648 (formerly known as *S. albus* BA3972A) and related strains.[40] It is a structural analogue of L-tryptophan and is a potent competitive inhibitor of the bacterial TrpRS as shown for *E. coli*.[41] It was patented by Pfizer in 1965, however further development was halted because indolmycin is relatively inactive against commonly occurring pathogens. Moreover it has been found that indolmycin inhibits catabolism of tryptophan in rat liver.[20a] The narrow spectrum of activity may be partly attributed to its hydrophobic nature which restricts its uptake by pathogens.[42] However, efforts have been invested to enhance the hydrophilicity of indolmycin but without success.[43] Recently, indolmycin has been described by Takeda Pharmaceuticals (Japan) as TAK-083 and was claimed to have potent activity against *H. pylori* (MIC 0.008-0.031 μg/mL).[44] In addition, Takeda Pharmaceuticals have patented TAK-083 containing formulation for the prevention and treatment of disease associated with *H. pylori* infections.[20a] However,

certain strains of *S. aureus* are found to develop high level resistance to indolmycin by a point mutation (H43N) in TrpRS, whereas naturally occurring *Streptomycetes coelicolor* A3(2) are resistant to indolmycin due to the presence of auxiliary TrpRS1.[41b]

Borrelidin

Borrelidin (**1.10**, Figure 1-5) was first isolated from *Streptomyces rochei* in 1949.[45] It has a unique structure and consists of a 18 membered polyketide macrocycle and has a broad-spectrum of antibacterial activity.[46] It is a potent but non-selective inhibitor of ThrRS which limits its clinical use as an anti-infective agent.[47] Moreover, resistance to borrelidin has been developed by over-production of ThrRS or by a single point mutation in the ThrRS gene.[48]

Cispentacin and icofungipen

Initially, cispentacin (**1.11**, Figure 1-5) was isolated from *Bacillus cereus* in 1989 and subsequently from *Streptomyces setonii*.[49] It is a cyclic β-amino acid derivative and is a weak inhibitor of ProRS.[50] It is actively transported by the amino acid permease leading to high intracellular levels in several fungi. It is an orally active antifungal agent. As it is a weak inhibitor of ProRS, efforts have been invested by Bayer AG in search of a new cispentacin analogue with improved oral activity and safety. During their efforts BAY 10-8888 (**1.12**, Figure 1-5) was identified as the most active compound of the series.[51] Although still based on cispentacin, BAY 10-8888 has a different mode of action. It acts as a potent inhibitor of fungal IleRS. The compound was then licensed to GlaxoSmithKline (formerly known as PLIVA) and renamed as PLD-118. Icofungipen is its generic name.[52] Analogous to cispentacin, icofungipen is also actively transported by the amino acid permease and accumulates in several fungi up to 200-fold of the extracellular concentration. Although low mycologic eradication rates were observed in HIV-positive patients as compared to fluconazole, icofungipen showed good efficacy and high tolerability in phase I and II of clinical trials after oral administration.[19b]

Cladosporin

Cladosporin (**1.13**, Figure 1-5) is a secondary metabolite isolated from various fungal genera such as *Cladosporium*, *Aspergillus*, *Eurotium*, and *Chaetomium*.[53] It

belongs to the isocoumarin class and is reported to have antifungal, antiparasitic, insecticidal, antibacterial and anti-inflammatory activity. In 2012, Winzeler *et. al.* reported that cladosporin is a potent and selective inhibitor of the cytosolic LysRS of *Plasmodium falciparum* (IC_{50} = 61 nM) whereas only weak inhibitory activity was detected against human LysRS at high micromolar range.[53] Despite of high selectivity and potent activity against liver-stage infections, cladosporin could not progress to clinical development due to poor oral bioavailability, which is a key requirement for antimalarial therapy.

Agrocin 84

Agrocin 84 (**1.14**, Figure 1-5) is a natural antibiotic produced by *Agrobacterium radiobacter* K84 used for biocontrol of the plant tumor. It is an Leu-AMP analogue decorated with a D-glucofuranosyloxyphosphoryl group linked to the N6 of the adenine moiety via a phosphoramidate linkage enabling uptake of the compound by the pathogen. Once internalized, the compound gets cleaved to release the toxic moiety which can act as a competitive inhibitor of LeuRS. The toxic moiety likewise comprises a stable phosphoroamidate mimicking aa-AMP.[54]

Ascamycin

Ascamycin (**1.15a**, Figure 1-5) is another example of a natural nucleotide antibiotic produced by *Streptomyces* species. Although it closely resembles 5'-*O*-(*N*-L-alanyl)-sulfamoyl adenosine (AlaSA), it has a different mode of action. It acts by inhibiting polyuridylate-directed polyphenylalanine synthesis. It shows selective antibacterial activity against *Xanthomonas citri* and *X. oryzae* due to the dealanylating activity of the Xc-aminopeptidase present on the cell surface of *Xanthomonas*, whereas its dealanyl analogue (**1.15b**, Figure 1-5) is a broad-spectrum antibacterial agent.[55]

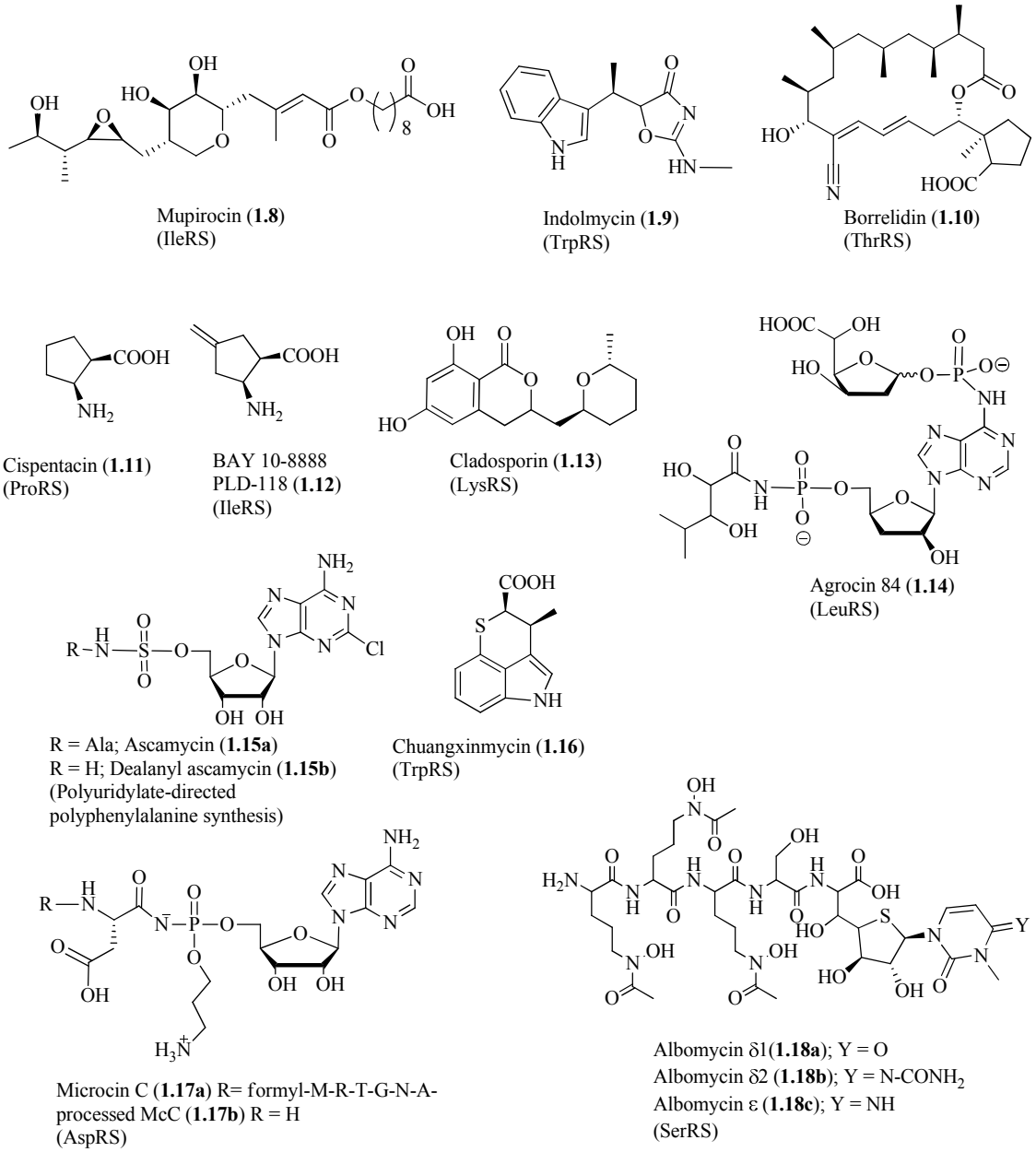


Figure 1-5: Chemical structures of few well-known aaRS inhibitors from natural source. (Note: Microcin C (**1.17**) and albomycin (**1.18**) will be discussed in detail in the next section 1.4 and 1.5.4 respectively.)

Chuangxinmycin

Chuangxinmycin (**1.16**, Figure 1-5) is a natural product isolated from *Actinoplanes tsinanensis*. As a consequence of its structural similarity with L-tryptophan and indolmycin, it also inhibits TrpRS (IC_{50} = 30 nM for *E. coli* TrpRS).[56] Chuangxinmycin is reported to have antibacterial activity against a number of Gram-

positive and Gram-negative bacteria under *in vitro* conditions. Moreover, early preclinical studies indicated that this compound exhibits efficacy against *E. coli* and *Shigella dysenteriae* in a mouse model.[56] Despite of early promising results, there is no further report on the clinical development of this compound.[20a]

1.3.2 Synthetic or aminoacylation reaction mechanism-based inhibitors

The early efforts in the search of antibiotics from natural source produced a number of aaRS inhibitors with a diverse set of chemical structures. However for various reasons, none of them (except mupirocin) reached the clinic. Therefore, during last decade, efforts have been dedicated to rational design of compounds based on either a substrate mimic or a non-hydrolysable mimic of aa-AMP (**1.19**, Figure 1-6).

Non-hydrolysable aa-AMP analogues

As described earlier, aminoacyl-AMP (**1.19**, Figure 1-6) is a key intermediate in the aminoacylation of tRNA. The aa-AMP has two to three orders of magnitude of higher affinity for the enzyme as compared to the substrate amino acid or ATP. Consequently, non-hydrolysable mimics of aa-AMP are expected to show excellent potency as compared to either substrate amino acid or ATP. The non-hydrolysable mimics of aa-AMP form the largest class of aaRS inhibitors. To date, the labile phospho ester linkage has been replaced with hydrolytically stable linkages such as aminoalkyl-phosphate (**1.20**),[39c, 57] ester (**1.21**),[58] amide (**1.22**),[58a] *N*-alkoxy-hydroxamate (**1.23**), hydroxamate (**1.24**),[58] aminoacyl-sulfamate (**1.25**),[25, 39c, 57a, 59] sulfamides (**1.26**),[39c] *N*-alkoxy-sulfamide (**1.27**) and *N*-hydroxy sulfamide (**1.28**).[60] The general structure for each class of compounds is depicted in Figure 1-6. Among them, only two classes of the compounds (aminoalkyl-adenylate and aminoacyl-sulfamates) closely mimic the negative charge density around the reactive acyl-phosphate of aa-AMP. Aminoalkyl-adenylate keeps the phosphate group but holds an aminoalkyl group instead of the aminoacyl whereas the aminoacyl sulfamate keeps the aminoacyl group but replaces the phosphate linkage with a sulfamate one. As a general trend, the aminoacyl-sulfamates have proved to be the most potent inhibitors with IC_{50} in the nanomolar range whereas aminoalkyl adenylate analogues are millimolar inhibitors of the corresponding aaRS *in vitro*. However, both of these analogues could not progress to clinical development due to their lack of selectivity (due to high structural similarity with the aa-AMP) and the poor *in vivo*

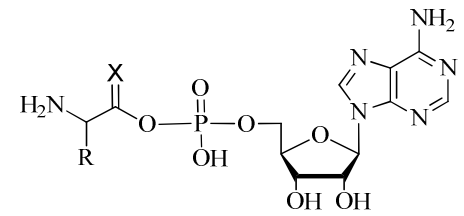
efficacy (due to poor cell penetration). As noted earlier in this section the aaSA analogues closely mimic the negative charge density as seen across the reactive acyl-phosphate, thus at physiological pH, all aaSA analogues exist as zwitterions with a negative charge on the sulfamate nitrogen and a positive charge at the α-amine of the amino acid. Because of their polar nature, these compounds can not diffuse easily through hydrophobic cell membrane. Therefore, most of the modifications of non-hydrolysable aa-AMP analogues are aimed to improve the selectivity and the *in vivo* efficacy.

Aryl-tetrazole containing aminoacyl-sulfamates

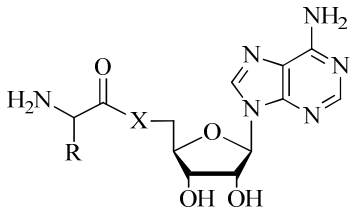
In 1998, Cubist Pharmaceuticals reported the synthesis and biological evaluation of a new class of aminoacyl-sulfamates where the adenine base was replaced with an aryl-tetrazole moiety and is linked to the ribose via a two carbon linker. These analogues proved to be potent and selective inhibitors of the corresponding aaRS with selectivity up to 3000-fold. Of the numerous compounds of this class, two aryl-tetrazole containing sulfamates are given in Figure 1-6 as examples. Despite of excellent *in vitro* potency and good selectivity, these analogues could not progress to clinical development due to their poor *in vivo* efficacy and high serum albumin binding.[8, 61] However, these analogues demonstrated that selective inhibition of the pathogen aaRS can be achieved by replacing the adenine base of the aaSA analogues with heterocycles which deviate significantly from adenine.

Mupirocin-based aaRS inhibitors

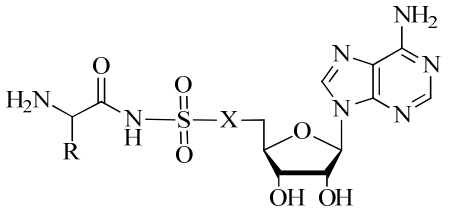
Mupirocin is the only aaRS inhibitor approved by the FDA for clinical use. However, its use is limited to topical application due to poor stability at physiological conditions. Therefore, efforts have been invested to improve the pharmacological properties of mupirocin. Hereto, a series of β-diketone derivatives have been synthesized and evaluated. The two most important analogues of this series are depicted in Figure 1-6. The compounds **1.31** (MIC of 1 μg/mL against *S. aureus*) and **1.32** (MIC of 0.5 μg/mL against *S. aureus*) displayed excellent antibacterial activity and improved pharmacological properties such as reduced serum binding. Both compounds were effective in treating mice infected with *S. aureus*, both after oral and subcutaneous administration.[39b] The compound SB234764 (**1.33**, Figure 1-6) is a rationally designed



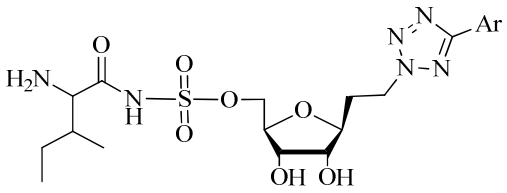
X = O; aminoacyl-AMP (**1.19**)
X = H, H; aminoalkyl-adenylate (**1.20**)



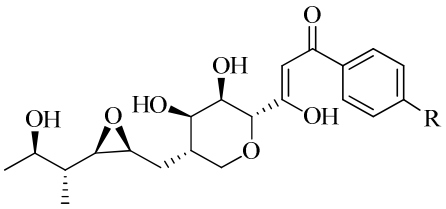
Ester (**1.21**); X = O
Amide (**1.22**); X = NH
N-alkoxy hydroxamate (**1.23**); X= NH-O
Hydroxamate (**1.24**); X = N-OH



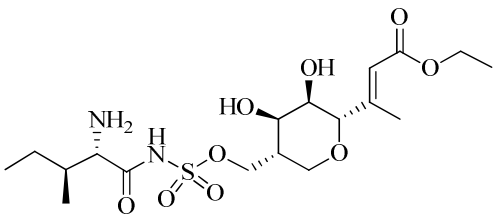
Sulfamate (**1.25**); X = O
Sulfamide (**1.26**); X = NH
N-alkoxy sulfamide (**1.27**); X= NH-O
N-Hydroxy sulfamide (**1.28**); X = N-OH



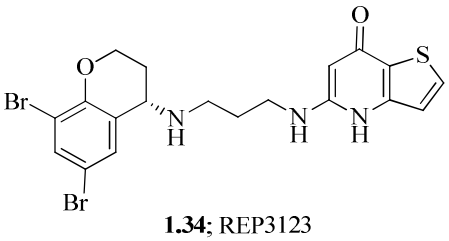
1.29; (CB 168); Ar = -C_6H_5
1.30; (CB 432); Ar = -C_6H_4-O-C_6H_5



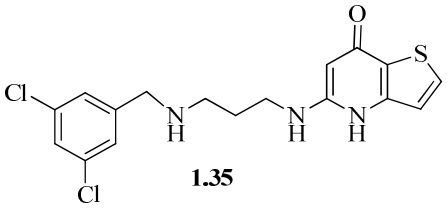
1.31; R = OCH_3
1.32; R = F



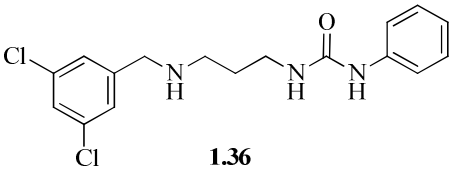
1.33; SB-234764



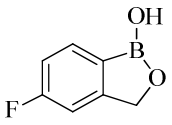
1.34; REP3123



1.35



1.36



1.37

Figure 1-6: aa-AMP (**1.19**), its non-hydrolysable analogues and few representative synthetic analogues as aaRS inhibitors.

femtomolar inhibitor of IleRS with K_d <0.01 nM. The compound combines the structural features of mupirocin (**1.8**, Figure 1-5) and isoleucyl-sulfamoyl adenosine (**1.25**, general structure for aaSA, Figure 1-6).[39a, 39c] Besides these analogues, several other modifications of mupirocin have been attempted but none of them was developed into clinically useful antibiotics.

MetRS inhibitors

In 2009, a novel series of diaryldiamine derivatives have been reported as bacterial MetRS inhibitors. These compounds are originally developed by GlaxoSmithKline and Replidyne for treatment of *S. aureus* and *Clostridium difficile* infections. As outlined by Critchley *et al*.[62] one of the analogue of this series, REP3123 (**1.34**, Figure 1-6) was active against 108 clinical isolates with MIC range of 0.5-1 μg/mL. REP3123 proved to be a strong and selective inhibitor of *C. difficile* MetRS with a calculated inhibition constant (K_i) of 0.02 nM with a selectivity of > 1000-fold for bacterial MetRS over its human orthologs. Later, these leads were further optimized by Buckner and colleagues in their efforts to fight trypanosomiasis.[63] A new series of diaryldiamines was uncovered, selectively inhibiting the bloodstream form of *Trypanosoma bruci* at low nanomolar concentration (**1.35**, Figure 1-6). The aminoquinolone heterocycle was further replaced with substituted arylurea derivatives to improve the bioavailability of these analogues (**1.36**, Figure 1-6).[64]

AN2690: an editing site inhibitor of LeuRS

Recently, Anacor Pharmaceuticals reported a series of substituted benzoxaborole derivatives for treatment of fungal infections, especially onychomycosis (nail fungal infection) and other cutaneous infections caused by fungi, yeast, molds or dermatophytes. AN2690 (**1.37**, Figure 1-6) is the most active compound of the series. Unlike other aaRS inhibitors, AN2690 is a non-competitive editing site inhibitor of LeuRS and acts by trapping the $tRNA^{Leu}$ through covalent bonding of boron with the 2'- and 3'-hydroxyl function of the 3'-terminal adenosine.[9a] The compound is claimed to penetrate through the nail bed by virtue of its physicochemical properties. It is a broad-spectrum antifungal agent with MIC 0.06-1 μg/mL. Unlike other antifungal agents, AN2690 has minimal affinity for keratin and the presence of 5% keratin does not affect its MIC value. Clinical

studies on human nails showed that the compound penetrates 250 times better than a conventional antifungal agent like ciclopirox.[65] AN2690 showed good efficacy and safety in phase I and II clinical trials and is now in phase III of clinical development for the treatment of onychomycosis.

1.4 Microcins

Microcins are ribosomally synthesized peptide antibiotics produced by the family of *Enterobacteriaceae* mainly *E. coli* strains of fecal origin.[66] Microcins are usually small molecules with molecular weight less than 10 kDa.[66b] Microcins were formerly called 'colicins' as they both are produced by the same family of bacteria, have the same mode of action and both are encoded by plasmid. However, microcins are differing from the colicins in many aspects like molecular weight and the mode of production and secretion out of the producing cells. Microcins are low molecular weight compounds usually less than 10 kDa whereas the colicins are much bigger (from 25-80 kDa). Colicin production is induced by the SOS system whereas microcins are produced when the bacteria approaches the stationary phase (the nutrients are depleted and cells become starved).[66b, 67] Until now, only 11 microcins have been identified and these microcins are MccB17, McC7, McE492, McJ25, McL, McV (also known as ColV), McD93, McH47, McI47, McM, Mc24.* However only the first six microcins have been structurally characterized.[68] In addition, based on the post-translational modifications, microcins are classified into two classes, modified and unmodified microcins. The unmodified microcins are usually polypeptides with a molecular weight ranging from 8 to 10 kDa and examples include McE492, McL and McV. In contrast, the modified microcins are usually smaller peptides with a molecular weight <5 kDa. These microcins are extensively modified post-translationally and examples include McB17, McC7, McJ25, McD93, McH47, McI47, McM and Mc24.[69] Being an aaRS inhibitor and relevant to this thesis, only microcin C has been discussed here.

1.4.1 Microcin C (McC)

Microcin C (McC, **1.17a**, Figure 1-5) is a nucleotide-peptide antibiotic produced by some *E. coli* strains during the stationary phase of growth. It is the smallest known microcin and consists of a modified AMP attached to the α-carboxyl group of aspartic acid through an *N*-acyl-phosphoramidate linkage. The later amino acid itself is at the C-

terminal end of a formylated heptapeptide.[70] The phosphate group of AMP is further decorated with a aminopropyl group.[66a] Microcin C displayed a broad-spectrum of antibacterial activity, being active against many Gram-negative and Gram-positive bacteria.[71] Previously, McC51 and McC7 are believed to be different microcins, however detailed NMR and mass spectrometry studies in combination with biochemical assays revealed that McC7 and McC51 are identical and thereafter both are referred to as McC.[70, 72]

1.4.2 Biochemical synthesis of McC

In *E. coli*, the plasmid carrying the *mccABCDEF* gene cluster determines the production, maturation, and extracellular export of McC and also provides self immunity.[73] Biosynthesis of McC begins with the translation of a 21 bp-long gene, *mccA* which is among one of the shortest protein-coding bacterial genes known. The heptapeptide product of *mccA* (formyl-MRTGNAN) undergoes extensive modifications as shown in Figure 1-7. The polypeptide product of *mccB* gene (MccB) catalyzes the attachment of heptapeptide to AMP in a two steps process, each requiring one molecule of ATP. In the first step, the nucleophilic attack of the α-carboxylate of Asn7 on the α-phosphate of ATP leads to formation of a heptapeptide-succinimide via heptapeptide-AMP. Similarly, in the second step, the nucleophilic attack of the succinimide nitrogen on the α-phosphate of ATP gives a reactive intermediate which upon hydrolysis gives the heptapeptide attached to AMP through an *N*-acyl-phosphoramidate linkage. The reaction results in conversion of Asn7 to Asp7 with the nitrogen linked to AMP.[74]

*Some authors prefer to use Mcc abbreviations for microcin. For e.g. Microcin C7 can be abbreviated as MccC7 or McC7

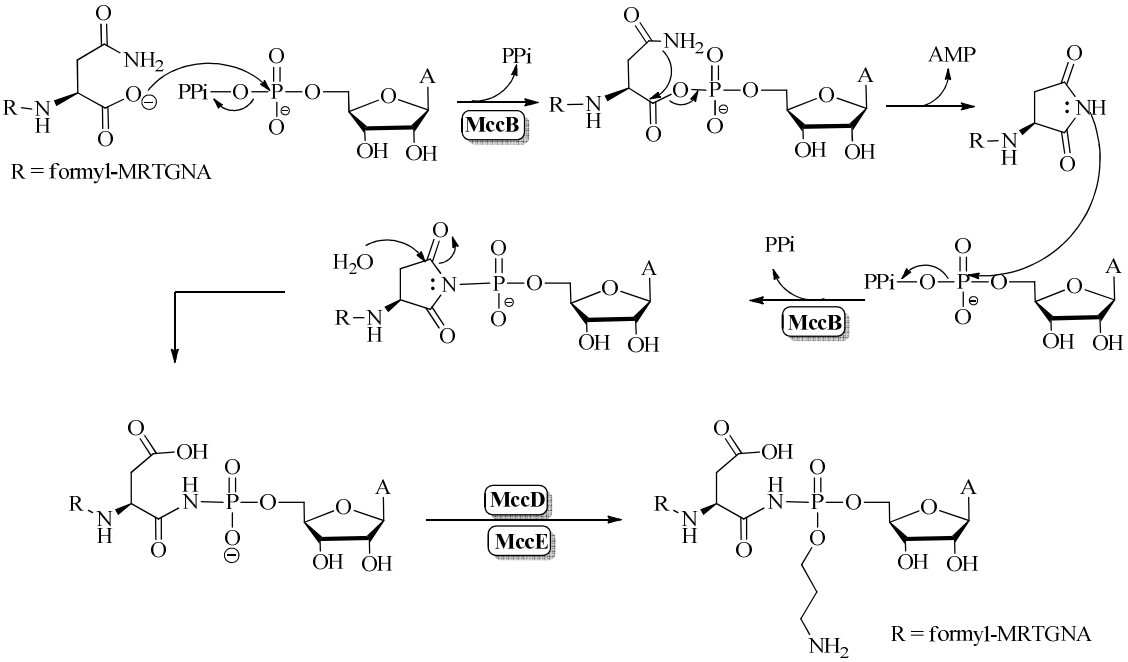


Figure 1-7: Synthesis and maturation of microcin C by MccB, MccD and MccE.

Following formation of the correct phosphoramidate linkage, the heptapetide adenylate is further modified by interplay between MccD and the *N*-terminal end of MccE which together responsible for the attachment of 3-aminopropyl to the phosphate moiety. MccD shows signature motifs as found in S-adenosylmethionine and MccE is similar to the 5'-phosphate-dependent decarboxylase. Therefore, it is hypothesized that the attachment of the 3-aminopropyl group may also follow a similar pathway, in which MccD may be responsible for transfer of a 3-amino-3-carboxyl group onto the heptapeptide-adenylate, followed by MccE catalyzing a decarboxylation yielding mature microcin C.[66a] It has been found anyhow that the 3-aminopropyl group increases the antibacterial activity by 10-fold (Figure 1-7).[75]

1.4.3 McC: Trojan-horse inhibitor

Mature microcin C is actively taken up by sensitive cells via YejABEF transporter. The McC peptide part is crucial for the recognition by the YejABEF transporter. Although the biological function of YejABEF and the natural substrate for the transporter is not known, it is believed that the YejABEF transporter may be involved in the transport of oligopeptides (containing *N*-terminal formyl-methionine) inside the

cells. In analogy, it has been observed that the same transporter contributes to the virulence of *Salmonella* and in addition Δ*yej* mutants became sensitive to antimicrobial peptides (AMPs).[76] Moreover, it has been shown that McC analogues with a shorter peptide length (less than six amino acids) are not recognized by the YejABEF transporter and formyl-methionine at the *N*-terminal increase the recognition by the transporter.[77] Once internalized, McC is processed by peptide deformylase followed by one of the broad-specificity aminopeptidases pepA, pepB or pepN to release the toxic moiety which is a non-hydrolyzable analogue of aspartyl-adenylate and inhibits AspRS. The mature McC does not inhibit the *in vitro* aminoacylation reaction and processed McC does not inhibit the growth of sensitive cells up to millimolar concentrations. Thus McC acts as a Trojan-horse antibiotic where the hexapeptide facilitates the transport of mature McC.[78]

1.4.4 Mechanisms of McC resistance or self immunity against McC

As AspRS of McC producing cells can be easily inhibited by processed McC, there must be mechanisms to ensure that McC production is not detrimental to the producing cells. One such mechanism involves MccC, a superfamily efflux pump encoded by the *mccC* gene and responsible for export of mature McC out of producing cells. It has been observed that deletion of *mccC* is dentrimental to the producing cells whereas over-expression of *mccC* alone provides resistance to McC sensitive cells. These observations indicate that MccC can provide resistance to internally produced or externally added McC.[70, 72, 75]

A Trojan-horse mechanism of action of McC further suggests that the accumulation of processed McC inside the producing cells is unavoidable; however the producing cells remain viable. Hereto, the internally produced processed McC is detoxicated by two different mechanisms. The first mechanism involves the acetyl-CoA-dependent acetyltransferease activity of the C-terminal of MccE protein ($MccE^{CTD}$). $MccE^{CTD}$ specifically acetylates the α-amino group of aminoacyl-nucleotides and does not depend on the nature of the amino acid nor the bond between the amino acid and the nucleotide nor the nature of the nucleotide (purines or pyrimidines). However, intact McC lacking the formyl group is not a substrate for MccE.[79] Acetylation of the α-amino of aspartate of processed McC may prevent its interaction with AspRS which provides the molecular basis for self immunity provided by MccE. The MccE acetyltransferase shares

homology with the bacterial N^{α}-acetyltransferases of the Rim family which are responsible for acetylation of ribosomal protein.

MccF is a serine protease which cleaves the carboxamide bond between the Asp7 residue of intact or processed McC and the nucleotide which confers the second line of defence mechanism against internally accumulated processed McC. Unlike to MccE, MccF is rather specific for aspartate or glutamate containing aminoacyl-nucleotides. It has been observed that MccF could not hydrolyse the phosphoramidate bond found in agrocin 84 or the amide bond found in albomycin.[80]

1.4.5 McC-based analogues as potential antibiotics

Microcin C is an attractive and versatile platform to design biologically active peptide-adenylates. Indeed the McC peptide moiety has been coupled to various aaSA analogues (Figure 1-8) which are evaluated for antibacterial activity. It has been found that these analogues also act as Trojan-horse inhibitors and growth inhibitory activities were comparable to native McC. However, the activities of the compounds containing a non-polar amino acid (Ile, Leu, Val, Ala, etc) at the seventh position proved slightly lower.[81] In addition, compounds lacking a formyl group at the *N*-terminal showed less active as compared to native McC but more active than McC lacking formyl and the aminopropyl group.[82] Deviation from McC structure could be quite extensive and McC analogues with different peptide moieties can be obtained by site-specific mutagenesis of *mccA* gene, or by chemical or enzymatic synthesis. It has been observed that the compounds with a shorter peptide chain (less than six amino acids) were poorly recognized by the YejABEF transporter. Replacing the *N*-terminal methionine with another amino acid abolishes the activity. Exclusion of Arginine at position 2 results in a drastic decrease in potency of the compound.[77] The nucleotide part also plays a vital role in the recognition by the YejABEF transporter. The compounds with an aryl-tetrazole moiety substituting for the adenine base apparently were not recognized.[83]

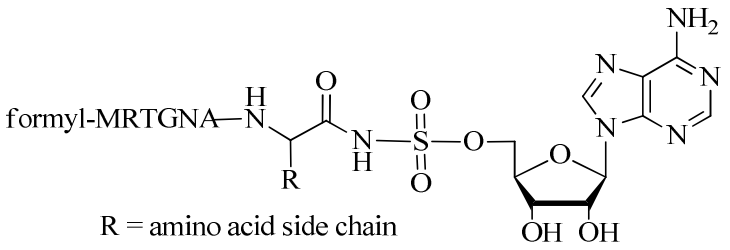


Figure 1-8: General structure for McC-based synthetic analogues (fXaaSA) targeting different aaRS. (fXaaSA, where X = MRTGNA, aa = amino acid).

1.5 Limited permeability of the outer membrane: a hurdle in antimicrobial therapy

As mentioned earlier, due to the emergence of multi-drug resistant pathogens, there is an urgent need for new anti-infective agents.[84] There are several mechanisms of bacterial resistance such as increased production of enzymes that inactivate/metabolise drugs, mutations leading to target alteration, efflux of the drug out of the bacterial cell and most important the limited permeability of cells. In most of Gram-positive bacteria, the cell wall consists of multilayer of peptidoglycan which forms the rigid structure and provides mechanical strength to the cell. On the contrary, the cell wall of Gram-negative bacteria consists of one or few layers of peptidoglycan, the periplasmic space and the outer membrane. The outer membrane of Gram-negative cell consists of lipopolysaccharides (LPS), lipoproteins and phospholipids which provides main barrier to many antibiotics. The cell envelop permeability barrier is particularly strong in Gram-negative pathogens like *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and other Gram-negative bacteria.[1b] These species are mainly associated with pneumonia, bacteraemia, and lung infection in cystic fibrosis. *P. aeruginosa* is well known for its intrinsic resistance due to its rigid, less diffusible cell membrane and its ability to develop multidrug resistance following therapy.[5a] Many antimicrobials are not effective against Gram-negative bacteria simply because they couldn't cross the cell membrane and reach the site of action.

There are several ways to overcome resistance due to limited permeability of the outer membrane of Gram-negative pathogens. One of them is the combination of drugs with an element acting on the outer membrane of the bacterial cell wall and altering its permeability, leading to increased concentration of drugs in bacterial cells. Examples of compounds acting on the outer membrane include pore forming antimicrobial peptides.

Alternatively, one can make use of the bacterial essential nutrient uptake system by a Trojan-horse mechanism.[5a] Therefore, an iron transport system is the most suitable for this Trojan-horse strategy as it is essential for all living organisms and plays a central role in the diverse biological functions.

1.5.1 Why iron?

It has been well documented that iron is an essential micronutrient for all forms of life except lactic acid bacteria.[6, 85] It plays an ubiquitous role in the variety of metabolic and informational cellular pathways. Several enzymes acting in the primary and secondary metabolic processes possess an iron containing cofactor. Iron is also an important part of the biological redox system. The reversible Fe^{2+}/Fe^{3+} redox pair makes iron an extremely versatile catalytic centre or enables its use as an efficient electron carrier.[86] Thus, iron is important for numerous biological processes such as photosynthesis, respiration, the citric acid cycle, oxygen transport, gene regulation, DNA biosynthesis etc.[6] Although iron is one of the most abundant elements in the earth crust, it does not occur in its biologically relevant ferrous form.[86a] Under aerobic condition ferrous ion is unstable. In aqueous environment, iron readily involves in the Fenton reaction to give ferric ion, and it further aggregates into an insoluble ferric hydroxide polymer. Therefore the availability of iron is limited to 10^{-18} M due to the low solubility of ferric hydroxide (Ksp = 10^{-38})[85] in aerobic conditions at physiological pH. In biological fluids, iron is tightly bound to high affinity proteins such as transferrin, lactoferrin or ferritin which further limit the growth of pathogens.[87] Bacteria require 10^{-6} M internal iron concentration to achieve optimal growth.[88] Virulence and even survival of infectious microbes largely depends on a key micronutrient like iron.[89] To circumvent this discrepancy, many microbes, plants and even higher organisms (i.e. octopi) synthesize and utilize very specific low molecular weight iron chelators called siderophores (previously called siderochrome).[85, 90]

1.5.2 Siderophores: a weapon in a battle for iron acquisition

As mentioned in the previous section, siderophores are low molecular weight iron chelators secreted by all microorganisms as part of an iron acquisition system. Under physiological condition, iron mainly exists in the ferric state (Fe^{3+}) which is relatively hard as compared to its reduced and biologically relevant ferrous (Fe^{2+}) form. In general,

siderophores have strong affinity for the ferric form. Therefore siderophores usually contain hard ligands bearing oxygen to coordinate with Fe^{3+}. However, upon internalization, Fe^{3+} is reduced to Fe^{2+}, in which the affinity of the siderophore for iron (II) is drastically reduced and iron is released as Fe^{2+}. Till date, more than 500 siderophores have been reported in the literature and based on their chemical structure (iron chelating moieties), they are classified into four main groups. These include α-hydroxycarboxylates, hydroxamates, catecholates and mixed ligand siderophores. The mixed ligand siderophore is usually comprised of combinations of two or all three aforementioned iron chelating moieties. Some general structures for siderophores are depicted in Figure 1-9. However, a detailed chemistry and biology of siderophores falls beyond the scope of this thesis and hence will not discuss here. Chemical synthesis of siderophores, their mechanisms of iron acquisition and transport across the cell membrane have been reviewed elsewhere.[91]

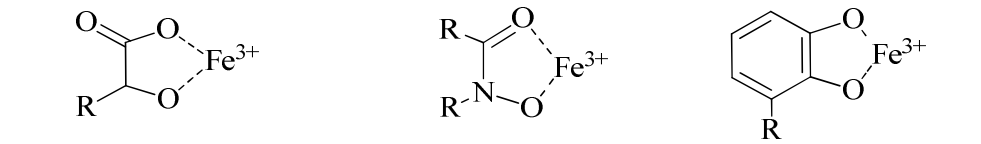


Figure 1-9: General structures of siderophores (shown with a single subunit of the iron chelating moieties).

1.5.3 Siderophore-mediated drug delivery: a Trojan-horse strategy

In summary, bacterial iron acquisition mechanisms are essential, efficient and vital for survival and virulence in the infected host. Therefore, the iron acquisition system is considered as an ideal target for anti-infective therapy. There are mainly three approaches which can be used to develop anti-infective agents which include (i) iron starvation via competitive chelation, (ii) siderophore biosynthesis inhibition[92] and (iii) Trojan-horse antibiotics using siderophore-mediated drug delivery. This dissertation deals with the third approach where the iron uptake system can be misused to circumvent the resistance associated with the low permeability barrier. Indeed, there are a few examples of natural siderophore-drug conjugates (SDCs) which include albomycin, ferrimycin, salmycin, etc. These antibiotics are actively taken up via the iron transport system

followed by metabolism to release the active moiety which elicits its toxic effect. Here, albomycin, salmycins and ferrimycins will be discussed as examples of sideromycins.

1.5.4 Albomycin

In 1951, albomycin was discovered by Gause and Brazhnikova.[93] However, the correct structure was not determined until 1982 by Benz *et al*.[94] Albomycin is a Trojan-horse antibiotic produced by *Streptomyces* strain ATCC 700974 as a mixture of closely related substances (**1.18a-c**, Figure 1-5).[95] In 1955, albomycin was used in the Soviet Union to treat bacterial infections in children.[93] It is a potent SerRS inhibitor and consists of a thioxylofuranosyl pyrimidine moiety linked to a siderophore (an iron chelator part) thus called sideromycin antibiotic. The siderophore part is comprised of a L-ornithine tripeptide-based trihydroxamate and is connected to the active moiety via an amide linkage.[96] Albomycins were classified as δ1, δ2 and ε based on the substituent at the cytidine base (**1.18a-c**). The siderophore part is responsible for the uptake of albomycin by virtue of its ability to form a complex with iron and is recognized by the iron channel. It is highly active against most of the Gram-negative and some of Gram-positive bacteria with MIC of 5 ng/mL against *E. coli* and 10 ng/mL against *Streptococcus pneumoniae*.

The transport of albomycin across the bacterial cell membrane has been studied in detail in *E. coli* K-12 and it was found that it is transported across the cell membrane by the same transport system as that of ferrichrome (a natural siderophore consisting of a cyclic hexapeptide with three glycines and three modified ornithines). The albomycin transport system is encoded by four genes *fhuABCD*. First albomycin is actively transported across the outer membrane of *E. coli* K-12 and delivered to the periplasmic space by the FhuA protein. In the periplasmic space it interacts with the FhuD protein and is shuttled to an ATP Binding Cassette (ABC) transport system embedded within the cytoplasmic membrane (FhuBC). Upon recognition of FhuD loaded with albomycin, FhuBC triggers the hydrolysis of ATP which in turn opens the channel through which albomycin is translocated to the cytoplasm.[97] Hereto, upon uptake, albomycin is metabolized by the serine peptidase (pepN) to release the toxic moiety which resembles seryl-adenylate and thus selectively inhibits bacterial SerRS. In case of *Salmonella typhimurium*, peptidase A is capable of releasing the toxic moiety.[98]

Resistance to albomycin has been observed through a point mutation in the FhuB gene which also results in loss of ability to recognize and transport ferrichrome and hence leads to decreased bacterial fitness.[95b, 99] Moreover, a single point mutation in the ATP binding domain of FhuC also confers resistance to albomycin.[100] Recently, albomycin proved to be effective in treating mice infected with *Streptococcus pneumoniae* or *Yersinia enterolitica*.[95b] However, the use of albomycin as a potential antibiotic is limited due to difficulties in obtaining sufficient amounts by isolation or chemical synthesis. Benz *et al.* synthesized an albomycin δ1 analogue replacing ribose for thioribose moiety. However, the newly synthesized analogue was to be inactive.[101] The synthesis of the ornithine tripeptide-based trihydroxamate was further improved by Miller by using indirect oxidation method.[102]

1.5.5 Biochemical synthesis of albomycin

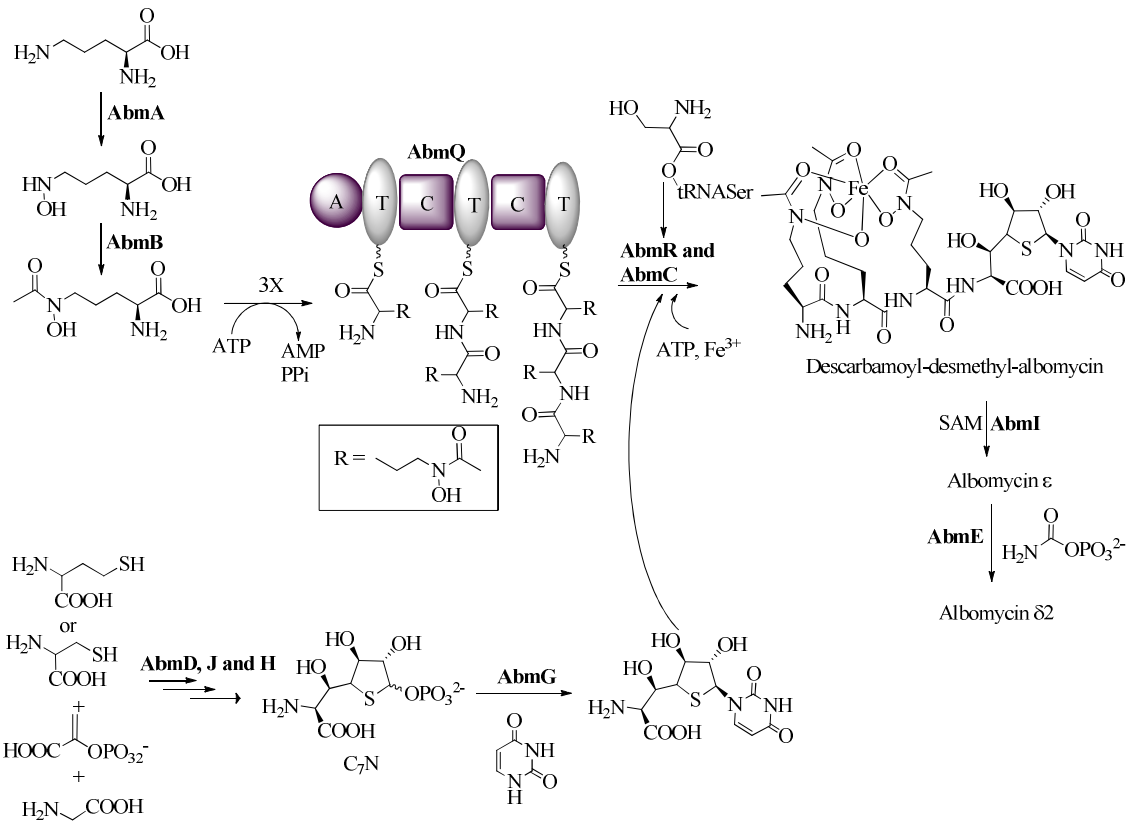


Figure 1-10: Proposed biosynthesis of albomycin in *Streptomyces* sp. ATCC 700974 (adapted from ref.[103])

Recently, biosynthesis of albomycin has been proposed based on detailed genetic and biochemical studies.[103] The gene cluster *abmABQ* is proposed to be responsible for

biosynthesis of the ferrichrome siderophore part of albomycin. AbmA is a flavin-dependent monooxygenase and responsible for synthesis of N^{δ}-hydroxylation of L-ornithine whereas AbmB is an *N*-acyltransferase and acetylates the N^{δ}-amino moiety using acetyl-CoA as a cofactor. Collectively AbmA and AbmB afford N^{δ}-acetyl-N^{δ}-hydroxy-L-orninthine (AHO) as a building block which is used by AbmQ, a non-ribosomal peptide synthetase (NRPS) for iterative condensation to yield the tripeptide siderophore. After off loading of siderophore tripeptide, AbmC and another ATP utilizing enzyme are proposed to be responsible for the formation of the remaining amide bonds including the amide bond between the unusual 4'-thioxylosyl amino acid and the serine.

Biosynthesis of the unusual 4-thioxylofuranosyl-cytidine moiety is very complex process and involves reactions such as oxidation, isomerisation, sulfur insertion and ring closure. Next, a typical *N*-glycosidic bond is formed by displacement of phosphate group at the anomeric carbon of thiosugar and is catalyze by AbmG. Finally, after attachment of the ferrichrome siderophore, the cytidine base is further modified by N3 methylation and N-carbamoylation catalyzed by AbmI and AbmE respectively. The biosynthetic pathway of albomycin is depicted in Figure 1-10.[103]

1.5.6 Salmycin

Salmycins (**1.42**, Figure 1-11) were isolated from cultures of *Streptomyces violaceus* DSM8286 by Vertesy and co-workers as a mixture of four different compounds. Salmycins showed potent activity against *Streptococci* and *Staphylococci* strains.[104] Because of stability reasons, salmycins B and C were isolated under acidic conditions whereas salmycin A and D were isolated strictly under neutral conditions.

Unlike albomycins, salmycins contain danoxamine (iron carrier) which is connected to the amino-disaccharide (active moiety) through an ester linkage. The exact mode of action of salmycin is unclear however; it is believed that they act as a protein synthesis inhibitor.[99] In 2000, Roosenberg and Miller postulated that upon reduction of iron, intramolecular cyclization confers the release of the active moiety in salmycins (Figure 1-11). In order to investigate the transport ability of danoxamine-drug conjugates and to elucidate the mechanism of drug release they coupled the danoxamine siderophore with commercially available antibiotics such as ciprofloxacin, triclosan and Lorabid® through either an ester or amide linkage.[105] They showed that the SDCs are actively taken up via the iron-channel. The order of antibacterial activity found was SDCs with an

ester linkage> parent drug> SDCs with amide linkage. The data clearly show that the release of the active moiety is the bottleneck in using siderophores as delivery agents. However, the proposed mechanism of drug release of salmycins remained to be confirmed.[105]

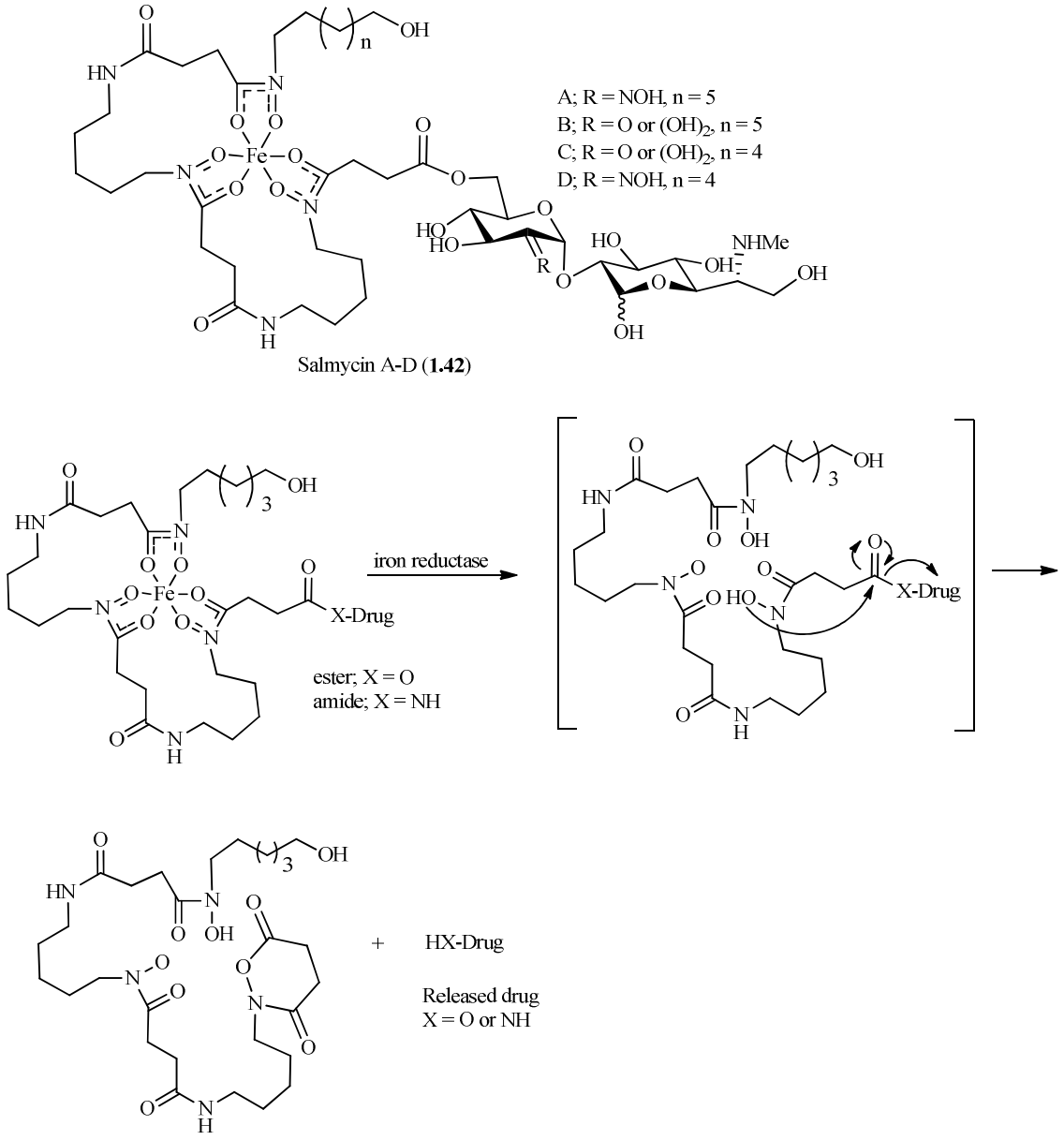


Figure 1-11: Structures of salmycin A-D and drug-release mechanism of danoxamine-drug conjugates. (adapted from ref.[105])

It has been observed that mutants of Gram-positive bacteria such as *S. pneumoniae* and *S. aureus* showed cross resistance among salmycins and albomycins. Moreover, the toxic effect of salmycin and albomycin can be nullified by addition of ferrichrome and ferrioxamine to the medium. These facts suggest that salmycin and albomycin presumably utilize the same uptake mechanism (iron-transport).[99] Albomycin has a broad-spectrum of activity and it is effective against Gram-positive and Gram-negative bacteria while salmycin is primarily active against Gram-positive bacteria. As compared to albomycin, salmycin proved to be more effective against multidrug resistant *S. aureus* (MRSA), especially with efflux mediated multidrug resistance (ERSA). However, comparative *in vivo* studies by Braun *et al* revealed that salmycin is less active, probably due to its labile ester linkage. Miller *et al.* reported synthesis of desferrisalmycin[104a], siderophore danoxamine[106] and several danoxamine-drug conjugates (manuscript in preparation according to ref.[105]).

1.5.7 Ferrimycins

Ferrimycin was isolated form *Streptomyces griseoflavus* (Strain ETH 9578) as a mixture of ferrimycin A and B.[107] Because of narrow-spectrum of activity, less attention has been given to ferrimycins. Ferrimycin A comprise of ferrioxamine B as an iron carrier connected to the active part via aminohydroxybenzoic acid bridge (**1.43**, Figure 1-12).

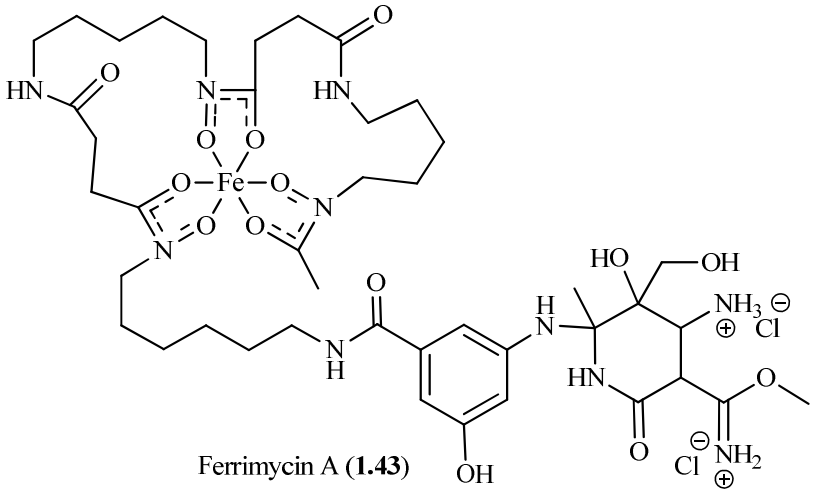


Figure 1-12 Structure of ferrimycin A

1.5.8 Synthetic siderophore-drug conjugates (SDCs): an application of Trojan-horse strategy

Inspired by the natural sideromycins, siderophore-mediated drug delivery has been demonstrated previously.[89, 108] Numerous synthetic SDCs of β-lactam antibiotics with different natural and synthetic siderophores have been prepared and evaluated for antibacterial activity. A few examples of such synthetic SDCs have been depicted in Figure 1-13. A commercially available antibiotic Lorabid® was coupled to a natural trihydroxamate siderophore (SDC **1.44**) and a mixed-ligand siderophore (SDC **1.45**). Both these SDCs exhibit mild antibacterial activity against Gram-positive and Gram-negative bacteria and showed delayed microbial growth as compared to the control. It has been found that mixed-ligand siderophore containing SDCs (**1.45**) appear to be able to use more than one iron-transport to deliver the active moiety to exert its toxic effect.[108b]

Analoguosly, Marvin *et al.* designed and synthesized a tris-catecholate siderophore with tripodal backbone and its conjugates with ampicillin (**1.46**) and amoxicillin (**1.47**). In iron-deficient media, both SDCs displayed significantly increased antibacterial activity against Gram-negative bacteria (MIC 0.05 to 0.39 μM) compared to their parent drugs (>100 μM) especially against *P. aeruginosa* while inhibitory activities of SDCs were impaired in iron rich media. The SDCs utilize energy-dependent iron-transport system for uptake which accounts for their enhanced antibacterial activity.[109]

In the same line, Mollmann and co-workers synthesized different catechol type SDCs and tested them for antibacterial efficacy. They further optimized the lead SDC structure with respect to the number of catechol moieties and the length of the linker separating the siderophore and the active moiety. A special feature of these SDCs is that the catecholate groups were masked either with acetyl, acetoxyl, methoxycarbonyloxy or with a cyclic dioxobenzoxazinyl group. The masking groups were claimed to cap the catechol groups thus preventing negative side effects.[5a, 110] Representative structures of the two SDCs (**1.48** and **1.49**) have been given in Figure 1-13.

Thus, overall the choice of siderophore, linker and drug all are very crucial for siderophore-mediated drug delivery. Iron channel mediated active transport may also reactivate drugs to which resistance has been developed by alteration in membrane permeability. Moreover, after penetration of SDCs across the outer membrane, the active moiety must be released to enable its inhibitory activity. Finally, siderophore alone or

SDCs may exert inhibitory activity simply by withholding iron which is essential for survival and virulence of pathogens.

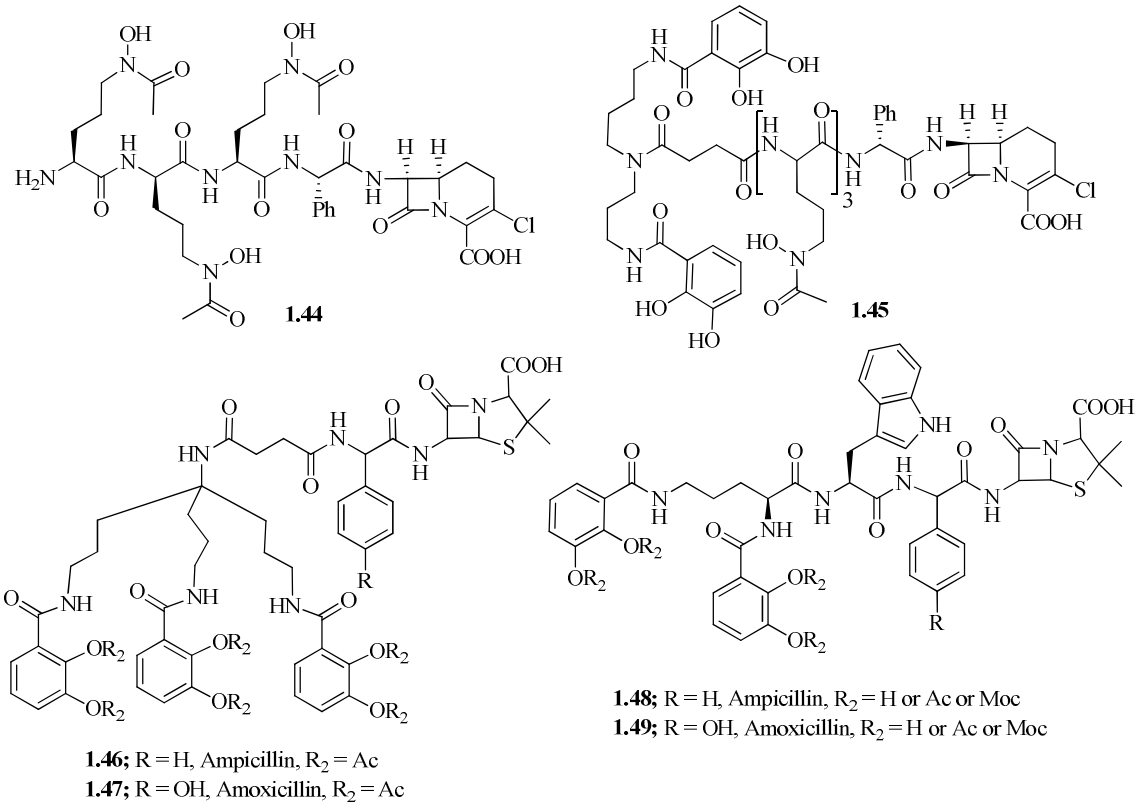


Figure 1-13: Selected examples of synthetic siderophore-drug conjugates (SDCs).

1.6 Hypothesis and objectives of this dissertation

Although, aaSA analogues proved to be excellent inhibitors of the corresponding aaRSs, further development was halted due to their poor *in vivo* efficacy and their lack of selectivity. Moreover, synthesis of aaSA analogues is cumbersome and low yielding due to a cyclic degradation product formed during their synthesis. Therefore, we set out specific objectives for our work as outlined below.

1.6.1. Siderophore-drug conjugates (SDCs): a Trojan-Horse strategy to improve the *in vivo* efficacy of aminoacyl-sulfamate derivatives.

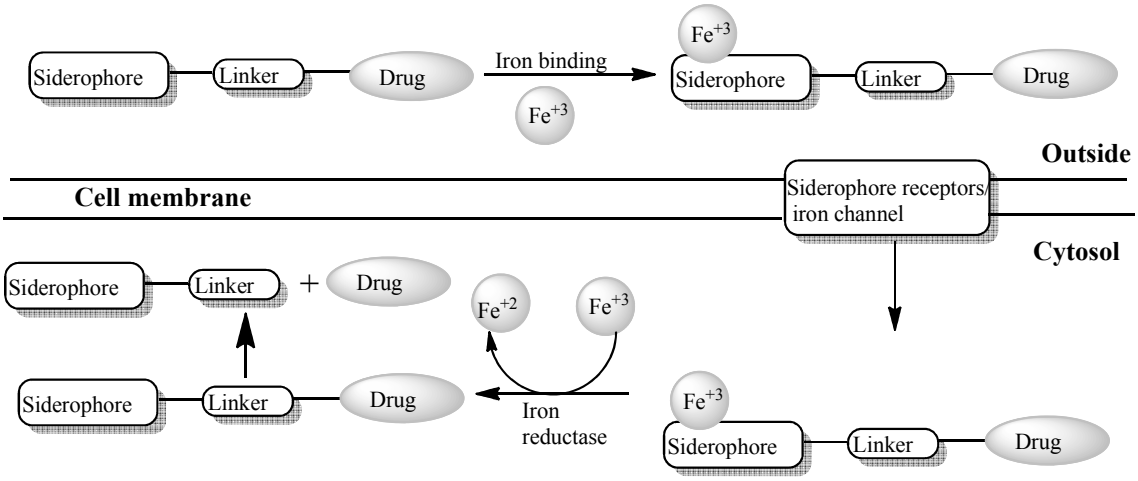


Figure 1-14: Siderophore-mediated drug delivery.

In a first part as described in chapter 2, we attempted to improve the *in vivo* efficacy of aminoacyl-sulfamate derivatives by combining them with a siderophore (an iron chelator part). In 1998, Cubist Pharmaceuticals reported a series of aryl-tetrazole containing aminoacyl-sulfamates as potent and selective inhibitors of the corresponding aaRSs. However, further development was halted due to their lack of cell-penetration and high serum albumin binding. We were interested to determine, whether the compounds reported by Cubist Pharmaceuticals when coupled to a siderophore part of albomycin or biscatecholate could function as a Trojan-horse inhibitors. Moreover, high selectivity of these analogues is an added advantage. Therefore, we hypothesized that when a siderophore-drug conjugate (SDC) would be presented to bacteria, it will be internalized by active transport using specialized transport (e.g. iron channel). Once inside the cell, the iron(III) should be reduced to iron (II) and the conjugate should be metabolized by broad-

specificity peptidases to release the active moiety and thus exert its antibacterial effect (Figure 1-14).

To test our hypothesis, the aryl-tetrazole containing sulfamates were coupled to the siderophore (trihydroxamate or biscatecholate) to yield SDCs which were evaluated *in vitro* as well as in cellular system for their potential antibacterial activity. The growth inhibitory properties of newly synthesized SDCs were determined in MHA, M63 and LB medium. To facilitate antibacterial evaluation and mechanism of action studies, the LB medium was supplemented with or without iron chelator. However, for unclear reasons, our attempts to synthesize triscatecholate siderophore (linear enterobactin analogue) failed. Moreover, we failed to get the desired aaSA containing trihydroxamate-based SDC due to their instability.

1.6.2. Determination of the pharmacophoric importance of the adenine base in aminoacyl-sulfamoyl adenosines (aaSAs).

Visual inspection of the compounds reported by Cubist Pharmaceuticals (**1.29** and **1.30**, Figure 1-6), and of albomycin (**1.18**, Figure 1-5) and mupirocin (**1.8**, Figure 1-5) revealed that these structures vary from aaSA analogues in having a heterocyclic base, a modified base or no base moiety at all as in mupirocin, respectively. This observation prompted us to investigate the pharmacophoric importance of the adenine base in aminoacyl-sulfamoyl-adenosines (aaSAa). Therefore, in second part as described in chapter 3, we synthesized and evaluated several isoleucyl-sulfamoyl nucleoside analogues with either uracil, cytosine, hypoxantine, guanine, 1,3-dideaza-adenine (benzimidazole) or 4-nitro-benzimidazole as the heterocyclic base (Figure 1-15). Based on the structure and antibacterial activity of microcin C, we also prepared their hexapeptidyl conjugates in an effort to improve their uptake potential.

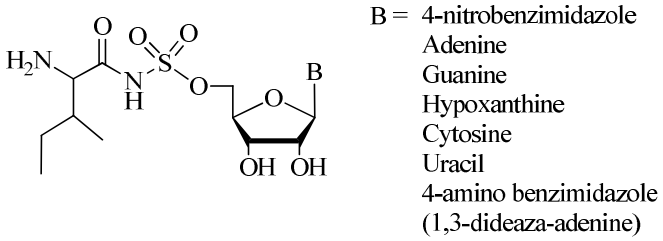


Figure 1-15: General structure of base substituted 5'-*O*-(*N*-isoleucyl)sulfamoyl nucleosides

1.6.3 5'-(*N*-aminoacyl)-sulfonamido-5'-deoxyadenosine: Attempts for a stable alternative to aminoacyl-sulfamoyl adenosines

Finally, it has been reported in the literature that during their synthesis sulfamoylated adenosines are prone to form cycloadenosine derivatives resulting in low yields for the different reactions and difficult purifications (Figure 1-16). Therefore, in the final part of this thesis, we looked for a stable alternative to aaSA analogues. Hereto, we hypothesized that deletion of the 5'-oxygen would render the C-5' less electrophilic and less prone to attack by N3 of the adenine moiety.

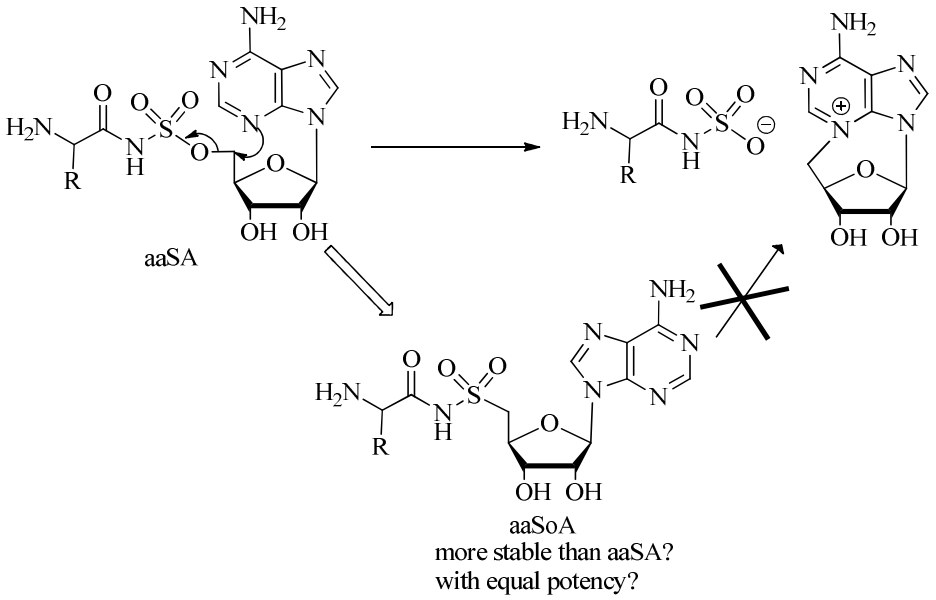


Figure 1-16: Cyclic adenosine derivative formed during synthesis of different aaSA analogues.

Thus a series of hitherto unknown aminoacyl sulfonamide (aaSoA) derivatives was prepared and evaluated *in vitro* as well as in a cellular system for their potential antibacterial inhibitory properties. Despite of numerous methods reported in the literature for synthesis of various sulfonamides. However, in our case, assembly of 5'-sulfonamido-5'deoxyadenosine (SoA) intermediate proved cumbersome but was finally realized using 1,3-dichloro-5,5-dimethyl hydantoin (DCDMH). These synthetic efforts will be described in chapter 4.

2 Siderophore-drug conjugates (SDCs): a Trojan-horse strategy to improve the *in vivo* efficacy of aminoacyl-sulfamate derivatives

Abstract

In 1998, Cubist Pharmaceuticals patented a series of aminoacyl tRNA synthetase (aaRS) inhibitors based on aminoacyl sulfamoyladenosines (aaSAs) where the adenine base was substituted by an aryl-tetrazole moiety linking to the sugar via a two-carbon spacer. Although being strong and specific inhibitors of bacterial IleRS, these compounds could not be pursued further due to low cell penetration and strong binding to serum albumin. In this chapter, we attempted to improve the *in vivo* efficacy of these compounds by combining them with an iron transport module called siderophore (either trihydroxamate or biscatecholate). We found that trihydroxamate-based SDCs of aryl-tetrazole variants still lacked antibacterial activity. However, these SDCs were readily processed by *E. coli* aminopeptidases with the release of toxic aaRS inhibitors. Hence, the lack of activity in the whole-cell assay was due to inability of the new compounds to be taken up by the cells. Possibly the absence of a nucleotide moiety in these analogues is responsible for facilitated transport for these compounds. Moreover, the biscatecholate-based SDC was not processed by the aminopeptidases and therefore failed to release the toxic aaRS inhibitor. However, for unclear reason, our attempts to synthesize the tris-catecholate siderophore (linear enterobactin) were not successful.

Part of this chapter was published earlier in an adapted form as:

"Microcin C and albomycin analogues with aryl-tetrazole substituents as nucleobase isoster are selective inhibitors of bacterial aminoacyl-tRNA synthetase but lack efficient uptake." Vondenhoff, G. H., Gadakh, B., Severinov, K., and Van Aerschot A., ChemBioChem, **2012**, *13* (13), p-1959-1969.[83]

2.1 Introduction

As mentioned in the previous chapter, increasing resistance to antibiotics is a major problem worldwide and provides the stimulus to search for new antibacterials or new cellular targets which are essential for the survival of pathogen.[111] One such target is aminoacyl-tRNA synthetases (aaRSs). These enzymes play an indispensable role in protein synthesis. These enzymes are responsible for the attachment of the correct amino acid to its cognate tRNA. Aminoacylation occurs in a two step process. In a first step the amino acid is activated to form aminoacyl-adenylate (aa-AMP) and in a second step is esterified to the 2'- or 3'-hydroxy group of the 3'-end of the respective tRNA.[9b, 9c, 11] To date numerous aaRS inhibitors have been discovered either from natural or synthetic origin.[12, 39c, 112] The best known aaRS inhibitors to date are aminoacyl sulfamoyl adenosines that are non-hydrolyzable isosters of the aminoacyladenylate (aa-AMP), which is the reactive intermediate in the aminoacylation of tRNA. Unfortunately these compounds could not be pursued further due to their lack of selectivity and poor *in vivo* efficacy.[25, 59a]

In 1998, Cubist Pharmaceuticals® reported synthesis and evaluation of a new series of aaRS inhibitors where the adenine base was replace with an aryl-tetrazole moiety and connected to the ribose through a two carbon linker. The tetrazole moiety is linked to either one or two five- or six-membered heterocycles. These analogues exhibit excellent *in vitro* activity with improved selectivity up to 3000 fold.[61] Some of the most important compounds, along with IleSA (**2.1**) are depicted in Figure 2-1 and their inhibitory properties are listed in Table 2-1. As can be seen, aryl-tetrazole-containing compounds **2.2** and **2.3** exhibit good activity against *E. coli* IleRS and poor activity against the human homologue (an especially profound difference is seen with CB168 (**2.3**)). Of the numerous compounds of this class synthesized and tested, only CB432 (**2.2**) showed moderate activity against a broad range of bacteria including *Staphylococcus aureus*, *S. epidermidis*, *S. saprophyticus*, *Streptococcus pneumonia*, *S. pyogenes*, *Enterococcus faecium*, *E. faecalis*, *Bacillus subtilis*, *Haemophilus influenzae*, *Klebsiella pneumoniae* and *Moraxella catarrhalis* with MIC values ranging between 2 and 100 μg/mL. In addition, CB432 (**2.2**) showed activity during treatment of mice infected with *S. pyogenes*. However, these compounds could not be pursued further as

Table 2-1 Comparison of IC_{50} values (in nM) for the selected Cubist Pharmaceuticals compounds along with Ile-SA against IleRS isolated from different microorganisms and human

SN	Compound	*S. aureus*	*E. faecalis*	*E. coli*	Human
1.	Ile-SA (**2.1**)	2.9	2	2	20
2.	CB 432 (**2.2**)	0.5	9	3.2	450
3.	CB 168 (**2.3**)	5	20	1.3	3000
4.	CB 286 (**2.4**)	4	10	1.3	524
5.	CB 360 (**2.5**)	4	4.4	12	190
6.	CB 447 (**2.6**)	0.5	8.6	1	570
7.	CB 628 (**2.7**)	3	6.3	27	455

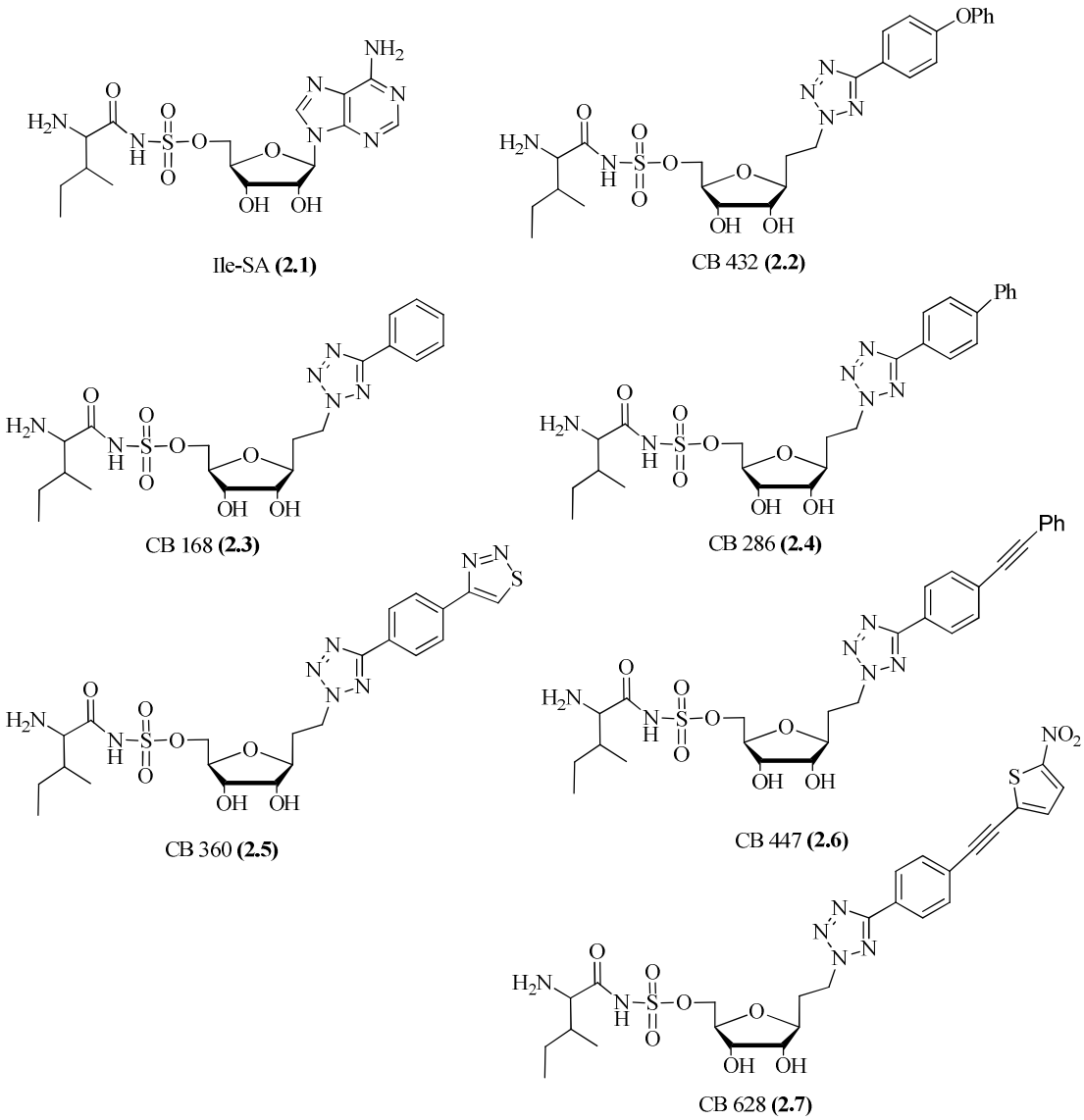


Figure 2-1: Chemical structures of a few aryl-tetrazole containing sulfamate derivatives along with Ile-SA

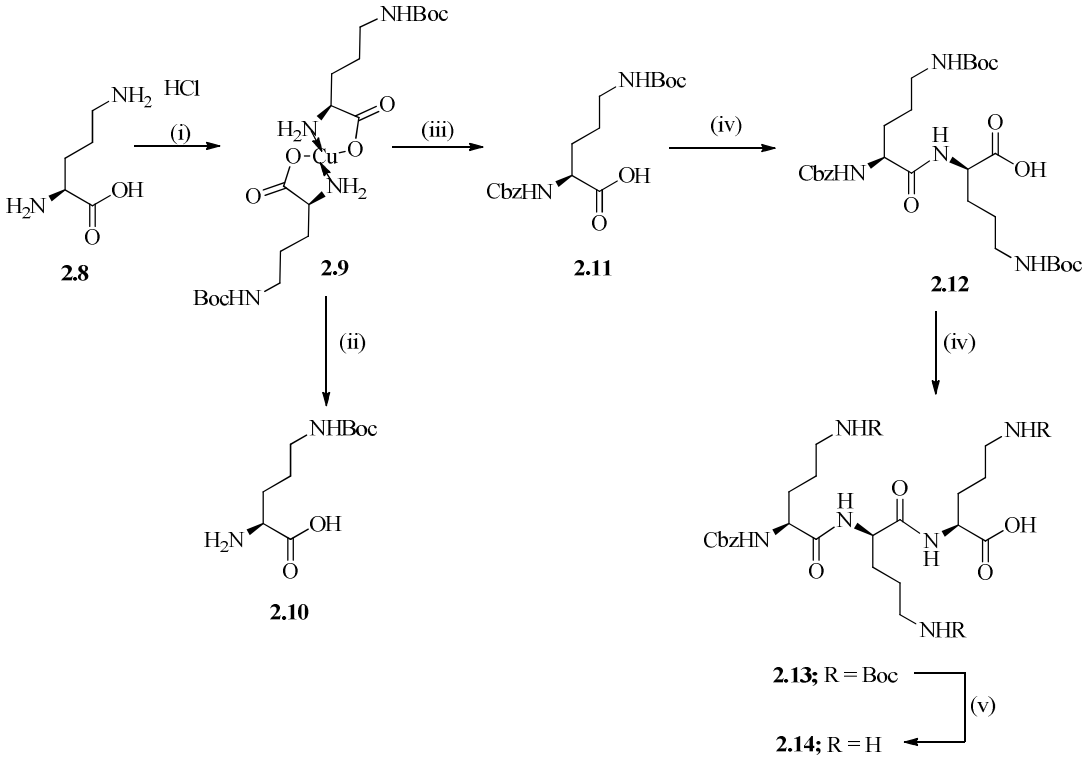
potential antibiotics due to their poor i*n vivo* efficacy and high affinity to serum albumin.[8] We were interested to determine, whether the compounds designed by Cubist Pharmaceuticals® when attached to the transport module of albomycin or to biscatecholate, could function as an aryl-tetrazole containing Trojan-horse inhibitor. To test our hypothesis, we concentrated on two different lead compounds, CB432 and CB 168 (**2.2** and **2.3**, Figure 2-1). The compound CB 432 was opted in view of its broad-spectrum of antibacterial activity, selectivity and moderate efficacy in an animal model, whereas CB168 was chosen for its great potential as a selective inhibitor of bacterial IleRS. Our hypothesis was that an aryl-tetrazole containing sulfamate coupled to a siderophore could be recognized by the iron channel. Once internalized, it could be metabolized by non-specific peptidases to release the active moiety and thus exert its antibacterial effect. Moreover, excellent selectivity of the parent analogues is an added advantage.

2.2 Results

2.2.1 Synthesis of L-ornithine-based trihydroxamate siderophore

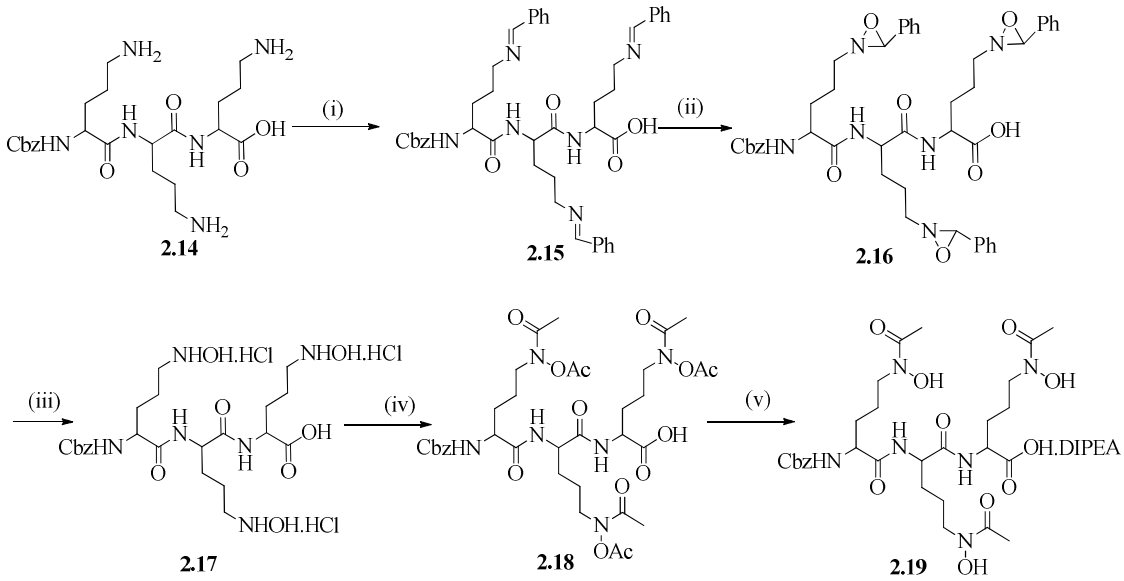
To test our hypothesis, we decided to couple the active moiety with the albomycin siderophore part as the latter is known to form a strong complex with iron and to be recognized by the iron-channel and to be metabolized by peptidases to release the active moiety.[96, 98] Therefore, we first focused our effort to the synthesis of L-ornithine tripeptide **2.14**. The required L-ornithine tripeptide was synthesized starting from L-ornithine.HCl **2.8** as outline in Scheme 2-1. Selective protection of the δ-amino group was achieved via Cu(II) complex of the L-ornithine followed by Boc protection of the δ-amino group using Boc anhydride in the presence of 2N NaOH to afford the bis(N^{δ}-Boc-L-ornithinato) Cu(II) complex **2.9** which upon copper removal gave compound **2.10**. Similarly, N^{α}-Cbz-N^{δ}-Boc-L-ornithine **2.11** was obtained in a one-pot reaction using 8-hydroxyquinoline as a copper sequestering agent and benzylchloroformate as a protecting agent in a mixture of acetone and water. This protocol omits a separate copper removal step.[113] As shown in Scheme 2-1, the required tripeptide was synthesized by employing an active ester mediated coupling between **2.10** and **2.11** as per literature procedure.[102] The active ester was prepared by reacting **2.11** with NHS and DCC in THF as a solvent. The resulting urea derivative was filtered off and the filtrate was used as such in the next

step. The active ester of **2.11** was reacted with **2.10** in a mixture of THF: water to provide dipeptide **2.12** in almost quantitative yield. Under the same coupling conditions, the dipeptide **2.12** was converted to tripeptide **2.13**. The N^{δ}-Boc groups were cleaved by using TFA:water to yield appropriately protected tripeptide **2.14**.[102] The desired siderophore (**2.19)** was synthesized starting from tripeptide **2.14** via its imine **2.15** which upon *m*-CPBA catalyzed oxidation afforded oxaziridine **2.16**. Acid catalyzed ring opening of the oxaziridine afforded hydroxylamine hydrochloride **2.17** which was then acetylated using acetic anhydride and potassium acetate buffer (pH 4) to provide compound **2.18**. Finally, the *O*-acetyl groups were removed by treatment with Hunig's base in methanol to yield the desired siderophore as a DIPEA salt **2.19** (Scheme 2-2).[102]



Scheme 2-1: Synthesis of L-ornithine tripeptide

Reagents and conditions: (i) $Cu(CH_3COO)_2 \bullet H_2O$, 2N NaOH, Boc_2O, acetone: H_2O, rt, 44 h; (ii) 8-hydroxyquinoline, acetone: water, 4 h; (iii) 8-quinoline $NaHCO_3$ acetone: H_2O, Cbz-Cl -10^{o} C to rt, 2 h; (iv) 1. DCC/ NHS, THF, rt, 10 h; 2. **2.10**, $NaHCO_3$, THF, water, rt, 6 h; (v) TFA: H_2O (5:2 v/v), rt, 3 h.

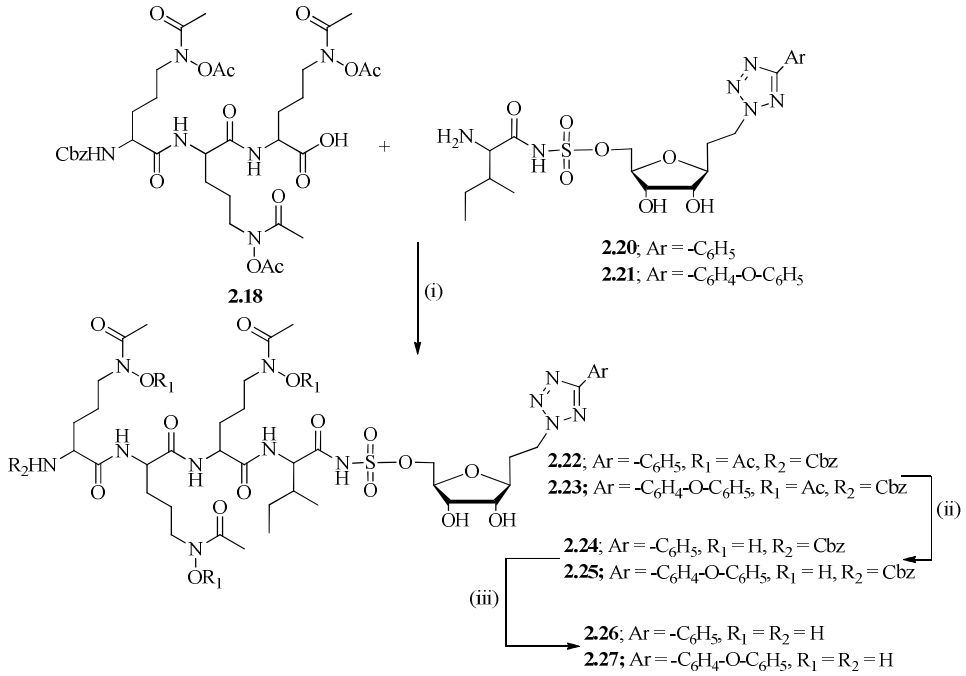


Scheme 2-2: Synthesis of L-ornithine-based trihydroxamate

Reagents and conditions: (i) Benzaldehyde, KOH, dry CH_3OH, molecular sieves 3 Å, rt, 16 h; (ii) *m*-CPBA, dry CH_3OH, 0° C, 4 h; (iii) 1. TFA:H_2O, CH_2Cl_2, 40-50° C, 15 min; 2. 1N HCl, rt, 2 h; (iv) Ac_2O, CH_3COOK/CH_3COOH, pH 4 buffer, rt, overnight; (vi) 6% DIPEA in CH_3OH, rt, overnight.

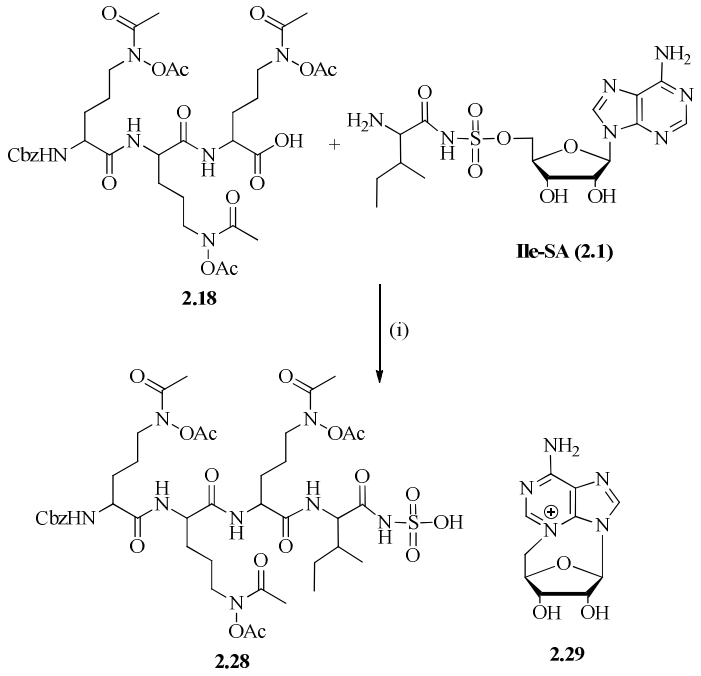
2.2.2 Synthesis of trihydroxamate-based siderophore-drug conjugates

Having the desired siderophore in hand, we attempted to couple the siderophore with an aryl-tetrazole containing sulfamate using HOBt, DIC and DIPEA as a base in DMF. However, we failed to get the desired product probably due to the competition between the α-amino group of isoleucine and the N^{δ}-hydroxyl function of the siderophore **2.19**. Therefore, protected siderophore **2.18** was coupled to the active moiety **2.20** under similar reaction conditions but the reaction was found to be low yielding. Moreover, diisopropyl urea, by-product of the reaction proved difficult to separate from the desired product. Therefore, we looked for other coupling conditions. The coupling reaction using HBTU, TEA in DMF afforded the coupled products **2.22** and **2.23** in good yield with straightforward purification. The coupled products were subjected to *O*-deacetylation using Hunig's base in methanol[102] followed by hydrogenolysis using Pd/C in a mixture of methanol: water to provide siderophore-drug conjugates **2.26** and **2.27** (Scheme 2-3). However, our attempts to synthesize the SDC of aminoacyl-sulfamoyl adenosine were unsuccessful due to formation of a cycloadenosine derivative as a degradation product (Scheme 2-4). Indeed, it has been reported in literature that sulfamoyl adenosines are prone to form cycloadenosine.[114]



Scheme 2-3: Synthesis of siderophore-drug conjugates

Reagents and conditions: (i) HBTU, TEA, dry DMF, rt, overnight (ii) 6% DIPEA in CH_3OH, rt, overnight (iii) Pd/C, CH_3OH:H_2O (4:1 v/v), H_2 atm. rt, 5 h.

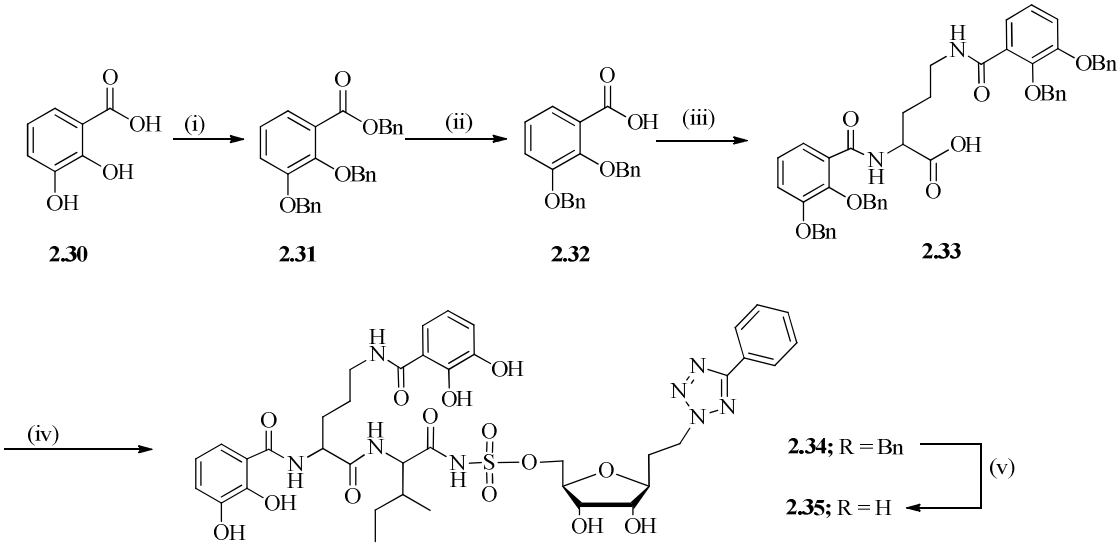


Scheme 2-4: An attempted synthesis of conjugate of triydroxamate siderophore with IleSA

Reagent and conditions: (i) HBTU, TEA, DMF, rt, overnight.

2.2.3 Synthesis of a biscatecholate siderophore and its drug conjugate

Beside the trihydroxamate-based siderophore, we also looked for an unnatural and relatively simple biscatecholate siderophore (**2.33**). This biscatecholate is known to promote the growth of bacteria which implies that it can form a complex with iron being recognized by the iron channel.[115] Moreover, SDCs of biscatecholate with β-lactam antibiotics proved to enhance antibacterial activity due to active uptake via the iron-channel.[110a] As shown in Scheme 2-5, the biscatecholate siderophore was synthesized starting from 2,3-dihydroxy-benzoic acid (2,3-DHB) **2.30**. Perbenzylation of **2.30** followed by hydrolysis of the benzyl ester afford compound **2.32** in good yield. Active ester mediated coupling of **2.32** with L-ornithine.HCl (**2.8**) afforded the protected siderophore **2.33** suitable for coupling with active moiety **2.20**. The HBTU mediated coupling of **2.33** with the active moiety **2.20**, was followed benzyl deprotection, providing siderophore drug conjugate **2.35** (Scheme 2-5).



Scheme 2-5: Synthesis of biscatecholate siderophore and its drug conjugate

Reagents and conditions; (i) $PhCH_2Br$, K_2CO_3, acetone reflux, 24 h; (ii) 5N NaOH, CH_3OH, reflux, 3 h; (iii) 1. DCC, NHS, THF, rt, overnight, 2. L-Orn.HCl (**2.8**), $NaHCO_3$, THF: H_2O, rt, 6 h; (iv) HBTU, TEA, dry DMF, rt, overnight (v) Pd/C, H_2 atm, CH_3OH: H_2O (4:1 v/v), rt, 2 h.

2.2.4 Synthesis of tris-catecholate siderophore

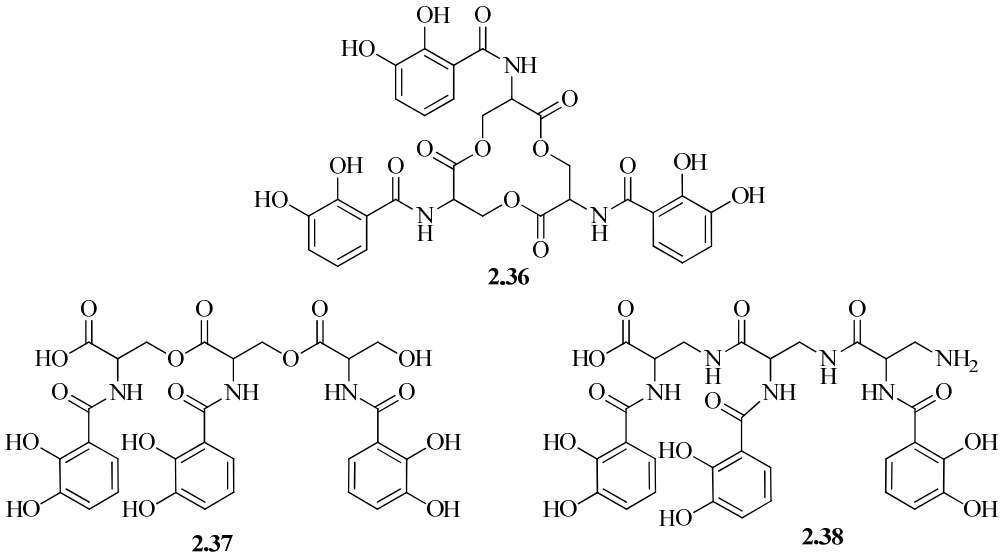
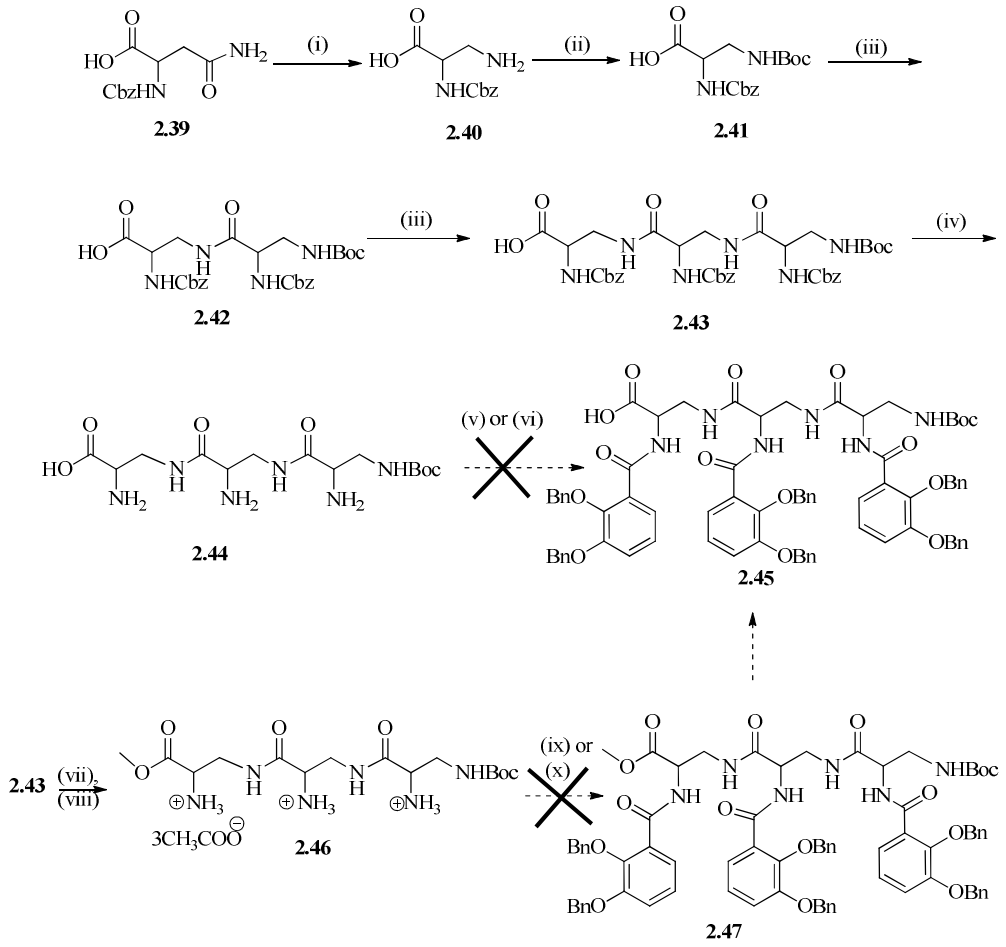


Figure 2-2: Chemical structure of Enterobactin (**2.36**), its linear analogue (**2.37**) and linear analogue with amide linkage (**2.38**).

In view of the disappointing results obtained with both the trihydroxamate and biscatecholate siderophores vide infra we continued to search for alternative. Enterobactin (Ent) (**2.36**, Figure 2-2) is an L-serine-based tris-catecholamide siderophore secreted by *E. coli*[116] and *Salmonella typhimurium*[117]. It is the strongest known iron chelator with an affinity constant[118] of 10^{52}. Because of its remarkable iron-binding property, several modified analogues (cyclic or acyclic) of the enterobactin have been synthesized and their iron-binding ability has been assessed.[91a] It has been reported in the literature that the linear enterobactine analogue (**2.37**, Figure 2-2) also has comparable affinity for iron (complex formation constant of $10^{46.5}$).[91a, 119] Therefore we decided to synthesize the linear analogue of Ent allowing the free carboxylic group to be used as a handle to attach the active moiety. We reasoned however that the ester linkages in the linear enterobactin analogue may not be stable enough as these can be cleaved by extracellular esterases. We therefore we decided to synthesize a linear enterobactin analogue comprising amide linkages (**2.38**, Figure 2-2). Synthesis of the tris-catecholate siderophore is outlined in Scheme 2-6. The N^{α}-Cbz-asparagine **2.39** upon Hoffmann rearrangement using phenyl-iodo diacetate (PIDA)[120] gave the β-amino-propanoic acid which upon Boc protection of

the β-amino group gave compound **2.41**.[121] An active ester mediated coupling of **2.40** with **2.41** afforded β-dipeptide **2.42**.



Scheme 2-6: Synthesis of tris-catecholate siderophore

Reagents and conditions: (i) PIDA, EtOAc:CH_3CN:H_2O (2:2:1), 15° C to rt, 5 h; (ii) Boc_2O 10% Na_2CO_3 1,4-dioxane, overnight, rt (iii) 1. DCC, NHS, THF, 0° C to rt, overnight; 2. **2.40**, $NaHCO_3$, THF: H_2O, rt, 6 h (iv) Pd/C CH_3OH, H_2 atm, 6 h; (v) 1. **2.32**, DCC, NHS, THF 0° C to rt, overnight; 2. $NaHCO_3$, THF:H_2O, 6 h; (vi) **2.32**, HBTU, TEA, DCM, rt, overnight; (vii) HBTU, TEA, CH_3OH, DCM, 2 h; (viii) Pd/C, THF: acetic acid (3:2), H_2 atm, 18h, rt; (ix) **2.32**, HBTU, TEA, DMAP, dry DMF, rt, overnight; (x) 1. **2.32**, oxalyl chloride, Toluene, 5° C, 30 min; 2. TEA, THF, 0° C to rt, 1 h.

Under the same coupling conditions, the β-dipeptide was converted to the β-tripeptide **2.43**. For unclear reasons however, we failed to couple the deprotected β-tripeptide **2.44** with 2,3-DHB (**2.32**) to yield appropriately protected siderophore **2.45** using either an active ester mediated coupling with DCC/NHS in THF: water or with

HBTU/TEA in DMF. Neverthless, both conditions afforded active ester of **2.32** in almost quantitative yield. Therefore, to facilitate final purification and to enhance solubility of the deprotected tripeptide **2.44**, the carboxyl terminal of the tripeptide was protected as a methyl ester and as shown in Scheme 2-6, deprotection of the N^{β}-Cbz group by hydrogenolysis afforded **2.46**. Acylation of the liberated amino groups was now attempted using either acyl chloride of **2.32** or active ester of **2.32** prepared in situ using using HBTU as a coupling agent. However, we failed again to get the desired product **2.47** which upon hydrolysis of the methyl ester would have yielded the desired tris-catecholate siderophore **2.45**.

2.2.5 Antibacterial activity of SDCs

2.2.5.1 Disc Diffusion method

The growth inhibitory activities of the SDCs **2.26** and **2.27** against *E. coli* strains (wt, ΔABN and ΔRim) and SDC **2.35** against *E. coli* wt were determined in MHA medium and M63 medium by disc diffusion method (for details see section 2.5.2.1). The growth medium was supplemented with and/or without 10 μg/mL of iron chelator such as 2,2'-dipyridyl or EDTA and with or without $FeCl_3$ as iron source. The overnight grown bacteria were incubated with different concentrations of inhibitor at 37^{o} C and the growth was observed over 18 h. None of the SDCs showed any growth inhibitory activity up to 5 mM (data not shown).

2.2.5.2 Micro broth dilution method

The lack of inhibitory activity was further confirmed by micro broth dilution test in a 96 well microtiter plate. Here, the ability of the new siderophore-drug conjugates (**2.26, 2.35**) to inhibit the growth of *E. coli* wt were determined by measuring the optical density reached by identical cell cultures in the wells of microtiter plates in the presence of various concentrations of respective inhibitors. To facilitate the activity evaluation and mechanism of action studies, the LB medium was supplemented with or without 2,2'-dipyridyl or alternatively with or without EDTA (10 μg/mL) as an iron chelator and with or without $FeCl_3$ (equimolar to SDC) as an iron source. Iron chelators were added to make the growth medium iron deficient in order to promote the uptake of SDCs. As can be seen in Figure 2-3, the SDC does not exert any growth inhibitory activity. However,

growth inhibition was observed in a medium containing iron chelator indicating iron is an essential element for growth and survival of bacteria. The maximum inhibitor concentration used was 500 µM which upon dilution gave 25 µM of final concentration.

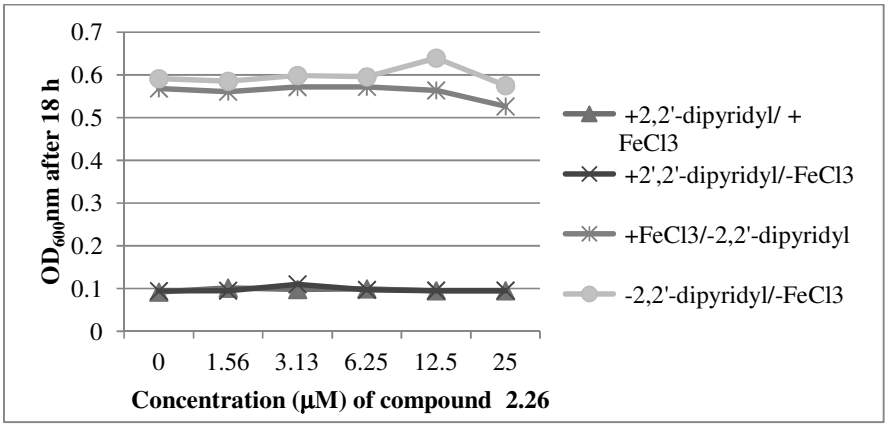


Figure 2-3: Whole-cell antibacterial assay for SDC **2.26** using micro broth dilution method.

In addition, the antibacterial activities of the plain toxic moieties and of their trihydroxamate siderophore conjugates were determined by monitoring the optical density of cell suspension of following strains: *S. aureus* ATCC 6538, *Staphylococcus epidermidis* RP62A (ATCC 35984), *Pseudomonas aeruginosa* PAO1, *Sarcina lutea* ATCC 9341 and *Candida albicans* CO11. Unfortunately, none of the analogues showed activity against the tested strains except compound **2.21**, which proved active against *S. aureus* and also showed low activity against *C. albicans* as found earlier by Cubist Pharmaceuticals. In contrast, SDC **2.27** showed hardly any activity against *S. aureus* and *C. albicans*. Hereto, the maximum concentration tested was 500 µM which upon broth dilution gave final inhibitor concentration of 25 µM (Figure 2-4).

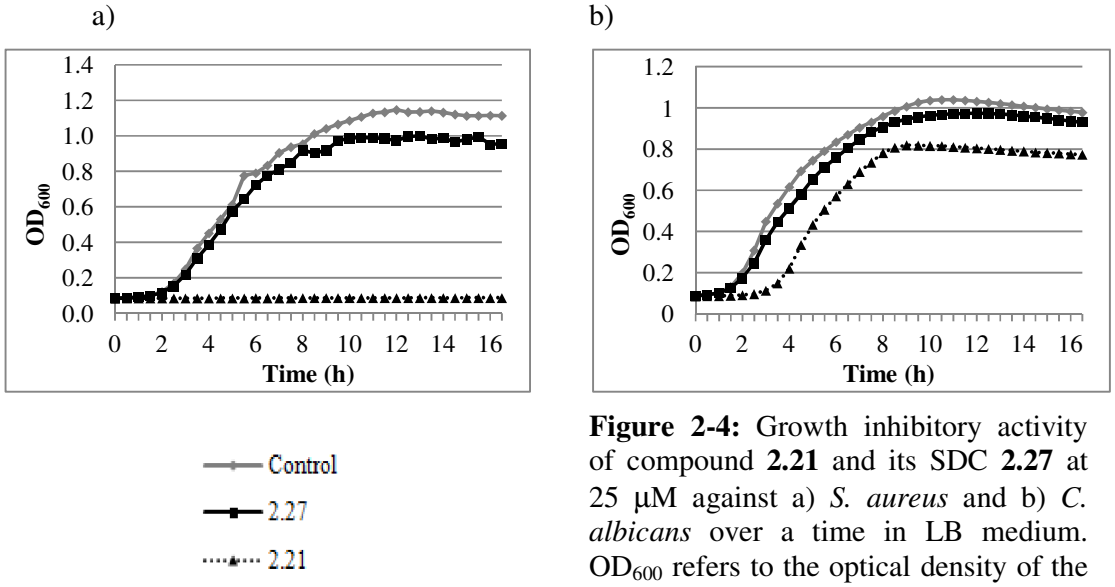


Figure 2-4: Growth inhibitory activity of compound **2.21** and its SDC **2.27** at 25 μM against a) *S. aureus* and b) *C. albicans* over a time in LB medium. OD_{600} refers to the optical density of the cell culture measured at 600 nm. .

2.2.5.3 In vitro evaluation of SDCs

To rationalize the reason(s) for this disappointing lack of antibacterial activity of the SDCs (**2.26** and **2.27**), *in vitro* analysis was performed and the ability of the active moieties and their SDCs to inhibit Ile incorporation using a S30 cell extracts from *E. coli* was determined (Figure 2-5). Both compounds and their SDCs showed nice inhibitory activity against IleRS in a cell extract. Only in a ΔpepABN (lacking peptidases pepA, pepB and pepN) extract SDCs were devoid of activity as anticipated. The results thus indicate that SDCs are processed in the expected way to release their active moiety which is able to inhibit IleRS (active moieties **2.20** and **2.21** do not require processing for target inhibition).

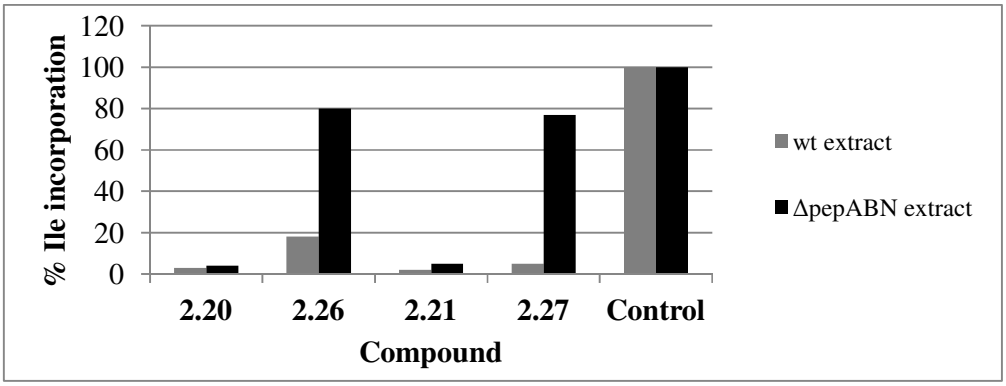


Figure 2-5: Inhibition of the *in vitro* aminoacylation reaction at 1.25 μM inhibitor concentration.

Likewise, SDC **2.35** was evaluated for its ability to inhibit IleRS under similar *in vitro* aminoacylation conditions. However, as can be seen in Figure 2-6, SDC **2.35** did not show any inhibitory activity against IleRS. Most likely reason for this inactivity could be the compound does not get processed by peptidases to release its active moiety within 15 min (incubation time). Therefore SDC **2.35** was incubated with the S30 cell extract and Ile incorporation was studied at various time intervals ranging from 5 min till 3 h. It has been found that the SDC **2.35** is not processed by peptidases to release the active moiety which in turn elicit inhibitory effect. However, even after 3h of incubation time, no inhibitory activity was detected, showing the active moiety could not be released (Figure 2-7).

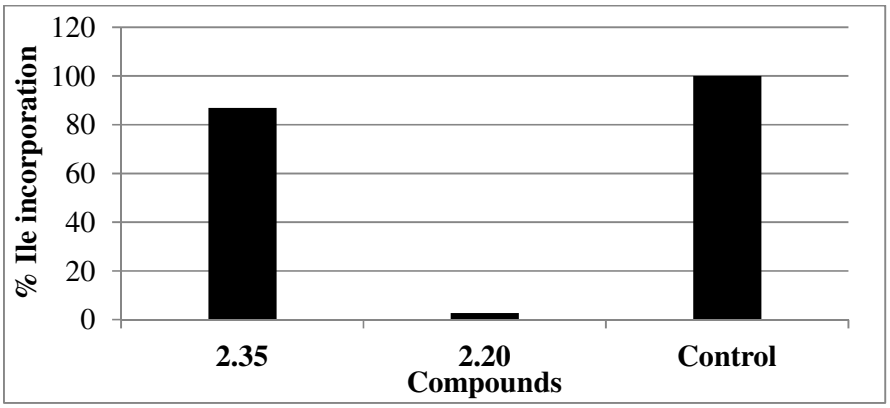


Figure 2-6: Inhibition of *in vitro* aminoacylation reaction at 2.50 µM inhibitor concentration.

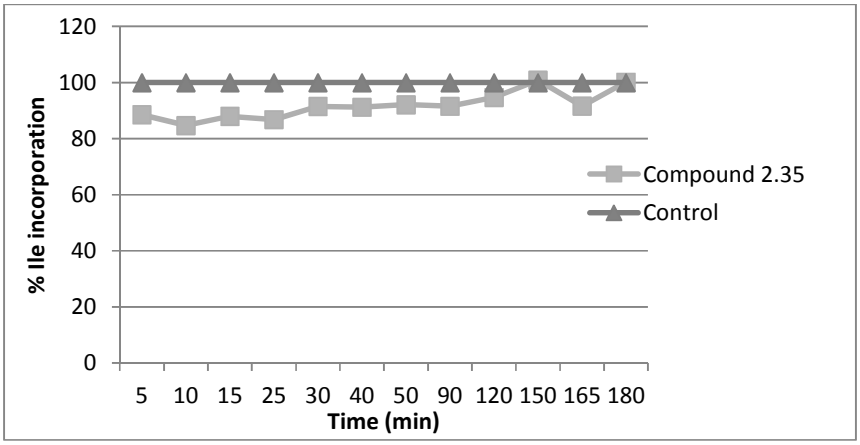


Figure 2-7: Biological acitivity of biscatecholate SDC **2.35** in S30 cell extract (*E. coli* wt) in function of time at 2.5 µM SDC concentration.

2.3 Discussion

2.3.1 Trihydroxamate-based SDCs

Siderophore-Drug Conjugates (SDCs) are meant only to facilitate the transport of the active moiety across the cell membrane. Therefore, the choice of the siderophore is determined by two important parameters. First, it should bind to iron with high affinity and should be recognized by the iron transport system. Secondly, upon internalization, it should get metabolized to release the active moiety. Therefore to prove our hypothesis, initial efforts were invested to synthesize L-ornithine tripeptide-based trihydroxamate as it is known from literature that it has strong affinity for iron (III), it is recognized by the iron channel and it can also undergo metabolism to release the active part.[96, 98] Although no antibacterial activity was observed in whole-cell screening, newly synthesized SDCs did show nice *in vitro* activity in cell extracts (except ΔABN cell extract). Hence, it can be concluded from this result that the SDCs are efficiently metabolized by cellular peptidases and thus release the active moiety which in turn inhibits the corresponding aaRS (IleRS), whereas release of the active moiety of synthetic SDC prodrug has often been marked as a main bottleneck.[105] Thus, failure of uptake is the main reason of the inactivity found for our SDCs which may be attributed to the structural differences between the albomycin and the newly synthesized SDCs. It has been observed by Vondenhoff *et al.* that when the McC signal peptide is coupled to aminoacyl-sulfamoyl adenosine, these conjugates are recognized by the YejABEF transporter and act as Trojan-horse inhibitors.[81, 83] However, McC signal peptide conjugates of aryl-tetrazole containing sulfamates were not recognized by the YejABEF transporter. Therefore, authors claimed that the YejABEF transporter may be selective for only peptidyl-adenylates or very closely resembling derivatives.[83] The same may be true for the SDCs that rely on FhuA and TonB complex for efficient uptake. Most probably insufficient recognition by FhuA or the Ton B complex which mediate the uptake of siderophores herein is the rate limiting step. Further studies need to resolve this issue. However, it is clearly shown that substitution of the adenine base with other heterocycles results in a lack of penetration of the bacterial membrane of all tested *E. coli* strains. Therefore, it would be highly desirable to investigate whether efficient uptake by iron channel can be obtained by modifying the two-carbon-linked aryl-tetrazole moiety. Our attempts to synthesize SDCs, by conjugation of the trihydroxamate siderophore with simple

aminoacyl-sulfamoyl-adenosines were not successful due to instability of these compounds (Scheme 2-4). It has been observed previously that aminoacyl-sulfamoyl adenosines are prone to form a cyclic adenosine derivative.[114] We observed a similar degradation product while synthesizing the trihydroxamate-based SDC of aminoacyl-sulfamoyl adenosine.

2.3.2 Biscatecholate-based SDC

With disappointing results obtained with the trihydroxamate-based SDCs, we looked for an unnatural and relatively simple siderophore. The biscatecholate siderophore was chosen for its straightforward synthesis. It is known to promote growth of *Pseudomonas aeruginosa* and *E. coli* strains indicating the siderophore forms complex with iron and is recognized by the iron channel.[115] However, our attempt to improve the *in vivo* efficacy of the aryl-tetrazole sulfamate was unsuccessful. It can be concluded from *in vitro* data (Figure 2-7) that the biscatecholate-based SDC was not processed by non-specific peptidases (pepA, pepB and pepN) and thus failed to release the active moiety. It has been observed previously by Kazakov *et al.* that mature microcin C was processed by these peptidases only after deformylation by peptide deformylase.[122] In other words, the braod-specificity peptidases cannot process microcin C if the *N*-terminal is protected or blocked. In retrospect, the *N*-terminal ornithine in our case is protected as a catecholamide which might be limiting factor to release the active moiety. However, when a $FeCl_3$ solution was added to a solution of SDC, it gave a purple colour indicating that the SDC has the ability to complex iron cations. Whether or not our SDC can be recognized by the iron transport system is less relevant, as in vitro tests already show the lack of inhibitory activity in cell extracts derived from *E. coli* wt.

2.4 Conclusions

Although all our efforts to improve the *in vivo* efficacy of an aryl-tetrazole containing sulfamate by uptake via the iron channel were unsuccessful, two important conclusions can be drawn from these results. First, trihydroxamate-based SDCs (**2.26** and **2.27**) are efficiently metabolized by cellular peptidases and thus release the active moiety which is often a bottleneck in siderophore mediated drug delivery. Therefore, the absence of antibacterial activity is mainly attributed to failure of uptake. This failure of uptake may be due to the loss of recognition at the iron channel which can be attributed to the

structural differences between the albomycin and the SDCs. Secondly, biscatecholate-based SDC (**2.35**) being protected at its *N*-terminal was not metabolized by cellular peptidases. Thus, failure to release the active moiety is the likely reason for the absence of antibacterial activity. Therefore apart from the nature of the active moiety, the choice of siderophore is very crucial for a successful siderophore mediated drug delivery. However, our efforts to synthesize siderophore conjugates of aaSA analogues as the active moiety were unsuccessful due to their chemical instability.

2.5 Experimental section

2.5.1 Materials and Methods

Reagents and solvents were purchased from commercial suppliers (Acros, Sigma-Aldrich, Bachem, Novabiochem) and used as provided, unless indicated otherwise. DMF and THF were of analytical grade and were stored over 4Å molecular sieves. All amino acids used were natural amino acids (L-amino acid). For reactions involving Fmoc-protected amino acids and peptides, DMF for peptide synthesis (low amine content) was used. All other solvents used for reactions were analytical grade and used as provided. Reactions were carried out in oven-dried glassware under a nitrogen atmosphere with stirring at room temperature, unless indicated otherwise.

^{1}H and ^{13}C NMR spectra of the compounds dissolved in $CDCl_3$, CD_3OD, DMSO-d_6 or D_2O were recorded on a Bruker UltraShield Avance 300 MHz, 500 MHz or 600 MHz spectrometer. The chemical shifts are expressed as δ values in parts per million (ppm), using the residual solvent peaks ($CDCl_3$: ^{1}H 7.26 ppm; ^{13}C, 77.16 ppm; DMSO: ^{1}H, 2.50 ppm; ^{13}C, 39.52 ppm; HOD: ^{1}H, 4.79 ppm; CD_3OD: ^{1}H, 3.31 ppm; ^{13}C, 49.00 ppm) as a reference. Coupling constants are given in Hertz (Hz). The peak patterns are indicated by the following abbreviations: br s = broad singlet, d = doublet, m = multiplet, q = quadruplet, s = singlet and t = triplet. High resolution mass spectra were recorded on a quadrupole time-of-flight mass spectrometer (Q-Tof-2, Micromass, Manchester, UK) equipped with a standard ESI interface; samples were infused in 2-propanol/H_2O (1:1) at 3 μL.min^{-1}.

For TLC, precoated aluminium sheets were used (Merck, Silica gel 60 F_{254}). The spots were visualized by UV light at 254 nm. Column chromatography was performed on ICN silica gel 60A 60–200 μm. Final products were purified using a PLRP-S 100Å column connected to a Merck-Hitachi L6200A Intelligent pump. Eluent compositions are expressed as v/v. Purity was checked by analytical HPLC on a Inertsil ODS-3 (C-18) (4.6 x 100 mm) column, connected to a Shimadzu LC-20AT pump using a Shimadzu SPD-20A UV-detector. Recordings were performed simultaneously at 254 nm and 214 nm.

Bis(N^{δ}-*tert*-butoxycarbonyl-L-ornithinato)Cu(II) complex (2.9)[113]

To a stirred solution of **2.8** (5.06 g, 30.0 mmol) in 2N NaOH (30 mL), a solution of copper acetate monohydrate (3.0 g, 15.0 mmol) in water (15 mL) was added followed by a solution of di-tert-butyl dicarbonate (8.51 g, 39.0 mmol) in acetone (60 mL). After 24 h, an additional portion of acetone (30 mL) was added and stirring was continued for another 20 h. The precipitate was collected and washed with a mixture of acetone:water (2:1v/v, 50 mL) and water (100 mL). The precipitate was dried *in vacuuo* to yield 6.06 g (11.54 mmol, 77%) of the title compound as a blue coloured solid. Melting point: 245-246^{o} C (lit 245.89^{o} C)
^{1}H NMR: The compound proved insufficiently soluble in various duteriorated solvents.

N^{α}-benzyloxycarbonyl-N^{δ}-*tert*-butoxycarbonyl-L-ornithine (2.11)[113b]

A suspension of **2.9** (5.40 g, 10.26 mmol) in acetone (20 mL) was intensively stirred for 15 min, water (20 mL) was added and stirring was continued for 10 min. Then, 10% aqueous Na_2CO_3 solution (40 mL) and 8-quinolinol (3.23 g, 22.57 mmol) were introduced. The mixture resulting after 1.5 h is called reaction mixture 1 and was subsequently used.

To a solution of *N*-hydroxysuccinimide (2.60 g, 22.57 mmol) in water (15 mL) placed in a separate flask, Na_2CO_3 (1.2 g, 11.29 mmol) was added, followed by acetone (20 mL) and the mixture was cooled to -5^{o} C. Benzyl chlorocarbonate (3.2 mL, 22.57 mmol) was introduced in portions to maintain this temperature. The whole mixture was left standing at -5^{o} C for a half hour with occasional stirring to give reaction mixture 2. This was poured into the stirred reaction mixture 1. After 1 h, the precipitate of copper quinolinate was filtered off and washed with water. The filtrate and washings were combined and acetone was evaporated. The residual aqueous solution was washed with dichloromethane (2x25 mL), and the DCM layer was discarded. The aqueous layer was acidified with saturated $KHSO_4$ to pH 2 and extracted with ethyl acetate (3x25 mL). The ethyl acetate layer was washed with brine, dried over sodium sulphate and evaporated to yield the desired compound as a pale yellow coloured oil. Acetone was added to the crude product and evaporated. Addition of acetone and evaporation was repeated twice to yield 6.23 g (17.04 mmol, 83%) the title compound as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.43 (s, 9H, Boc CH_3), 1.48-1.60 (br s, 2H, Orn-γ-CH_2), 1.62-1.76 (m, 1H, Orn-β-CH_2, Ha), 1.87 (br s, 1H, Orn-β-CH_2, Hb), 3.10 (br s, 2H, Orn-δ-CH_2), 4.18-4.40 (m, 1H, Orn-α-C*H*), 5.09 (s, 2H, CH_2-Ph), 5.7 (br s, 1H, CON*H*), 6.21 (br s, 1H, CON*H*), 7.32 (br s, 5H, Ph); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.4, 25.9 (Orn-γ-*C*H_2), 28.4 (Boc *C*H_3), 29.5 (Orn-β-*C*H_2), 39.8, 40.8 (Orn-δ-*C*H_2), 53.7 (Orn-α-*C*H), 67.0, 67.4 (*C*H_2-Ph), 79.6, 80.9 (Boc *C*(CH_3)$_3$), 128.0 (*ortho*-aromatic), 128.1 (*para*-aromatic), 128.5 (*meta*-aromatic), 136.2 (*ipso*-aromatic), 156.3, 156.4 (*C*ONH-Boc, *C*ONH-Cbz), 176.0 (*C*OOH); HRMS for $C_{18}H_{26}N_2O_6K$ ($[M+K]^+$) calcd: 405.1428 found 405.1413.

N^{α}-benzyloxycarbonyl-N^{δ}-*tert*-butoxycarbonyl-L-ornithinyl-N^{δ}-*tert*-butoxycarbonyl-L-ornithine (2.12)[102]

To a solution of **2.11** (2.2 g, 6.0 mmol) and *N*-hydroxy succinimide (760 mg, 6.6 mmol) in THF (20 mL) at 0^{o} C was added a solution of DCC (1.36 mg, 6.6 mmol) in THF (10 mL), The reaction was stirred at room temperature for 12 h. The resulting DCU was filtered off and washed with THF. The THF filtrate containing the active ester was used in the following reaction.

To a solution of **2.10** (1.53 g. 6.6 mmol) and $NaHCO_3$ (1.32 g, 15.72 mmol) in a mixture of water/THF (30 mL/40 mL) at room temperature was added the active ester obtained above and reaction was stirred for 6 h. The volatiles were evaporated, and the aqueous residue was diluted with ethyl acetate and acidified to pH 2 with saturated $KHSO_4$. Ethyl acetate layer was separated; the aqueous part was extracted further with ethyl acetate (50 mLx3). The combined ethyl acetate layers were collected, dried over sodium sulphate and evaporated. The residue was subjected to silica gel column chromatography (2.5% AcOH/EtOAc) to afford 2.81 g (4.84 mmol, 81%) of dipeptide **2.12** as a white solid.

^{1}H NMR (300 MHz, DMSO-d_6) δ 1.25-1.75 (m, 26H, 2xBoc CH_3, Orn-β-CH_2x2, Orn-γ-CH_2x2), 2.89 (br s, 4H, Orn-δ-CH_2x2), 3.95-4.05 (m, 1H, Orn-α-C*H*), 4.09-4.2 (br s, 1H, Orn-α-C*H*), 4.68-4.82 (m, 2H, CON*H*), 5.02 (s, 2H, CH_2-Ph), 7.25-7.40 (m, 5H, aromatic), 8.04 (d, 1H, *J* = 7.2 Hz, CON*H*, peptide); ^{13}C NMR (75 MHz, DMSO-d_6) δ 24.6 (Orn-γ-*C*H_2), 28.4 (Boc *C*H_3), 28.6 (Orn-β-*C*H_2), 51.9 (Orn-α-*C*H), 54.3 (Orn-α-*C*H), 65.5 (*C*H_2-Ph), 77.6 (Boc *C*$(CH_3)_3$), 127.8 (*ortho*-C, aromatic), 127.9 (*para*-C, aromatic), 128.5 (*meta*-C, aromatic), 137.1 (*ipso*-C, aromatic), 155.7 (*C*ONH Boc), 156.0 (*C*ONH Boc), 172.1 (*C*ONH Cbz), 173.6 (*C*OOH); HRMS for $C_{28}H_{43}N_4O_9$ ($[M-H]^-$) calcd: 579.3035 found 579.3058.

N^{α}-benzyloxycarbonyl-N^{δ}-*tert*-butoxycarbonyl-L-ornithinyl-N^{δ}-*tert*-butoxycarbonyl-L-ornithinyl-N^{δ}-*tert*-butoxycarbonyl-L-ornithine (2.13)[102]

To a solution of **2.12** (2.81 g, 4.84 mmol) and *N*-hydroxy succinimide (612 mg, 5.32 mmol) in THF (20 mL) at 0^{o} C was added a solution of DCC (1.1 g, 5.32 mmol) in THF (10 mL). The reaction was stirred at room temperature for 12 h. The resulting urea DCU was filtered off and washed with THF. The THF filtrate was used as such in the following reaction. To a solution of **2.10** (1.24 g. 5.32 mmol) and $NaHCO_3$ (1.06 g, 12.58 mmol) in a mixture of water/THF (30 mL/40 mL) at room temperature was added the active ester obtained above and the reaction was stirred for 6 h. The volatiles were evaporated, and the aqueous residue was diluted with ethyl acetate and acidified to pH 2 with saturated $KHSO_4$. The ethyl acetate layer was separated and the aqueous layer was extracted further with ethyl acetate (50 mLx3). The ethyl acetate layer was collected, dried over sodium sulphate and evaporated column purification afforded 2.70 g (3.40 mmol, 70%) of the tripeptide **2.13** as a white solid.

^{1}H NMR (300 MHz, DMSO-d_6) δ 1.30-1.72 (m, 39H, Boc-CH_3x3, Orn-β-CH_2x3 and Orn-γ-CH_2x3), 2.88 (br s, 6H, Orn-δ-CH_2), 3.90-4.05 (m, 2H, Orn-α-CHx2), 4.15-4.30 (m, 1H, Orn-α-CH), 5.02 (s, 2H, 2H, CH_2-Ph), 6.70-6.86 (m, 3H, CONH), 7.29-7.45 (m, 5H,aromatic), 7.67-7.76 (m, 1H, CONH), 7.92-8.02 (m, 1H, CONH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 26.1 (Orn-γ-CH_2), 26.2 (Orn-γ-CH$_2$), 28.4 (Boc CH$_3$), 29.6 (Orn-β-CH$_2$), 52.4 (Orn-α-CH), 52.8 (Orn-α-CH), 54.5 (Orn-α-CH), 65.5 (CH$_2$-Ph), 77.5 (Boc-C(CH$_3$)$_3$), 127.8 (*ortho*-C, aromatic), (*para*-C, aromatic), 128.5 (*meta*-C, aromatic), 137.2 (*ipso*-C, aromatic), 155.7 (CONH Boc), 156.0 (CONH Boc), 170.9 (CONH), 171.9 (COOH); HRMS for $C_{38}H_{62}N_6O_{12}Na$ ([M+Na]$^+$) calcd: 817.4318 found: 817.4276.

N^{α}-benzyloxycarbonyl-L-ornithinyl-L-ornithinyl-L-ornithine (2.14)[102]

A solution of tripeptide **2.13** (7.8 g, 9.8 mmol) in a mixture of TFA/water (50 mL/20 mL) was stirred at room temperature for 3 h. The volatiles were removed and the resulting residue was dissolved in water (200 mL). The aqueous solution was washed with ethyl acetate and was concentrated to give 8.19 g (9.8 mmol, 100%) of the **2.14** as a foam.

^{1}H NMR (300 MHz, D$_2$O) δ 1.60-2.0 (m, 12H, Orn-β-CH_2x3 and Orn-γ-CH_2x3), 3.01 (br s, 6H, Orn-δ-CH_2x3), 4.14 (br s, 1H, Orn-α-CH), 4.33 (br s, 2H, Orn-α-CHx2), 5.14 (s, 2H, CH_2-Ph), 7.43 (br s, 5H, Ph); ^{13}C NMR (75 MHz, D$_2$O) δ 22.8, 22.9, 23.0 (Orn-γ-CH$_2$), 27.4, 27.5, 27.7 (Orn-β-CH$_2$), 38.5 (Orn-δ-CH$_2$), 52.7, 52.9, 54.1 (Orn-α-CH), 66.8 (CH$_2$-Ph), 114.1, 118.0 (TFA α-CF$_3$), 162.4, 162.9 (TFA C=O), 172.7, 173.9 (CONH), 175.4 (COOH); HRMS for $C_{23}H_{39}N_6O_6$ ([M+H]$^+$) calcd: 495.2953 found 495.2935.

N^{α}-benzyloxycarbonyl-N^{δ}-acetyl-N^{δ}-O-acetyl-L-ornithinyl-N^{δ}-acetyl-N^{δ}-O-acetyl-L-ornithinyl-N^{δ}-acetyl-N^{δ}-O-acetyl-L-ornithine (2.18)[102]

To a solution of KOH (1.18 g, 21.03 mmol) in methanol (60 mL) at room temperature was added tripeptide **2.14** (4.0 g, 4.78 mmol), followed by benzaldehyde (2.2 mL, 21.03 mmol) and 3 Å molecular sieves. The reaction mixture was stirred at room temperature for 16 h. The molecular sieves were filtered off and washed with methanol. The filtrate was concentrated to give the crude imine **2.15** as a pale yellow oil which was used further without purification.

^{1}H NMR (300 MHz, CD$_3$OD) δ 1.63-1.97 (m, 12H, Orn-β-CH_2x3 and Orn-γ-CH_2x3), 3.59 (br s, 6H, Orn-δ-CH_2x3), 4.15-4.28 (m, 2H, Orn-α-CHx2), 4.35-4.47 (m, 1H, Orn-α-CH), 5.0-5.08 (m, 2H, CH_2-Ph), 7.24-7.34 (m, 5H, aromatic), 7.34-7.46 (m, 9H, aromatic), 7.64-7.75 (m, 6H, aromatic), 8.26-8.34 (m, 3H, Imine); ^{13}C NMR (75 MHz, CD$_3$OD) δ 26.3 (Orn-γ-CH$_2$), 26.4 (Orn-γ-CH$_2$), 28.9 (Orn-β-CH$_2$), 29.3 (Orn-β-CH$_2$), 29.9 (Orn-β-CH$_2$), 52.9 (Orn-α-CH), 54.2 (Orn-α-CH), 54.4 (Orn-α-CH), 59.6 (Orn-δ-CH$_2$), 59.7 (δ-CH$_2$), 60.2 (δ-CH$_2$), 66.0 (CH$_2$-Ph), 127.1, 127.3, 127.6, 127.7, 128.0, 128.5, 129.0, 130.3, 130.4, 134.0, 135.4, 135.5, 136.4 (aromatic), 162.5, 162.8, 162.9

(Imine *C*=N), 171.3 (*C*ONH), 173.1 (*C*ONH), 176.8 (*C*OOH); HRMS for $C_{44}H_{49}N_6O_6$ ($[M-H]^-$) calcd: 757.3719 found 757.3704.

To a solution of crude imine **2.15** (4.78 mmol) in methanol (40 mL) at 0^o C was added a solution of *m*-CPBA (70%, 7.07 g, 28.68 mmol) in methanol (20 mL) over 2 h. The reaction was stirred at 0^o C for additional 2 h. The resulting precipitate was filtered off and washed with methanol. The filtrate was concentrated at room temperature to give a pale yellow solid, which was partitioned between water (150 mL) and ethyl acetate (150 mL) following adjustment to pH 2 with 1N HCl. The aqueous layer was extracted once again with ethyl acetate. The combined extracts were washed with brine, dried over sodium sulphate, filtered, and concentrated to yield crude oxaziridine **2.16** as a pale yellow solid.

To a solution of crude oxaziridine **2.16** (4.78 mmol) in a mixture of TFA/water (50 mL/10 mL) at room temperature was added CH_2Cl_2 (60 mL). The solution was stirred at 50^o C for 15 min. The volatiles were removed to give a light yellow oily residue. To this residue were added CH_2Cl_2 (120 mL) and 1N HCl (180 mL). The mixture was stirred at room temperature for 2 h, and the layers were separated. The aqueous layer was washed with CH_2Cl_2 (50 mLx2) and hexane (50 mLx2) and concentrated to give crude hydroxylamine.HCl **2.17** which was used further without purification

To a solution of hydroxylamine.HCl **2.17** (4.78 mmol) in KOAc/HOAc buffer (pH 4) (15 mL) was added $KHCO_3$ (108 mg, 1.08 mmol). Acetic anhydride (2 mL, 1.98 mmol) was added drop wise at room temperature over a period of 1 h. The pH of the solution was maintained at 4.5-5.0 by addition of solid potassium bicarbonate during this process. The reaction mixture was stirred at room temperature overnight. The volatiles were removed to give an oily residue. To this residue were added 0.5N HCl (50 mL) and ethyl acetate (50 mL). The organic layer was separated and aqueous layer was extracted with ethyl acetate (25 mLx4). The combined organic layer was washed with brine, dried over sodium sulphate, filtered and concentrated to give the compound 1.75 g (3.85 mmol, 46% over 4 steps) as a colourless oil.

^{1}H NMR (300 MHz, CD_3OD) δ 1.60-1.70 (m, 12H, Orn-β-CH_2 and Orn-γ-CH_2x3), 2.0 (br s, 9H, N-OCOCH_3 CH_3x3), 2.20 (br s, 9H, N-COCH_3 CH_3x3), 3.71 (br s, 6H, Orn-δ-CH_2x3), 4.15 (br s, 1H, Orn-α-C*H*), 4.38 (br s, 2H, Orn-α-C*H*x2), 5.09 (s, 2H, CH_2-Ph), 7.25-7.40 (m, 5H, aromatic); ^{13}C NMR (75 MHz, CD_3OD) δ 18.2 (NCO*C*H_3), 20.2 (Orn-γ-*C*H_2x3), 24.3 (N-OCO*C*H_3), 29.8, 30.2 (Orn-β-*C*H_2), 48.2 (Orn-δ-*C*H_2), 53.4 54.1, 55.8 (Orn-α-*C*H), 67.7 (*C*H_2-Ph), 128.8 (*ortho*-C, aromatic), 129.0 (*para*-C, aromatic), 129.5 (*meta*-C, aromatic), 138.2 (*ipso*-C, aromatic), 158.4 (*C*ONH Cbz), 170.3, (*C*ONH), 173.2, 173.4, 173.7, 173.8, 174.5, 174.7 (*C*ONH N-*C*OCH$_3$ and N-O*C*OCH$_3$), 174.9 (*C*OOH); HRMS for $C_{35}H_{50}N_6O_{15}Na$ ($[M+Na]^+$) calcd: 817.3226 found 817.3215.

N^{α}-benzyloxycarbonyl-N^{δ}-acetyl-N^{δ}-hydroxyl-L-ornithinyl-N^{δ}-acetyl-N^{δ}-hydroxyl-L-ornithinyl-N^{δ}-acetyl-N^{δ}-hydroxyl-L-ornithine DIPEA salt (2.19)[102]

A solution of hydroxamate **2.18** (500 mg, 1.37 mmol) in a methanolic diisopropylethylamine solution (6%, 20 mL) was stirred at room temperature for overnight. The volatiles were removed to give 541 mg (1.37 mmol, quantitative) of light yellow oil.

^{1}H NMR (300 MHz, CD_3OD) δ 1.35 (d, 15H, *J* = 6.6 Hz, DIPEA C*H*$_3$x5), 1.58-1.87 (m, 12H, Orn-β-C*H*$_2$x3 and Orn-γ-C*H*$_2$x3), 2.04-2.14 (m, 9H, N-COC*H*$_3$x3), 3.19 (m, 2H, DIPEA C*H*$_2$-CH$_3$), 3.52-3.76 (m, 8H, Orn-δ-C*H*$_2$x3 and DIPEA C*H*-CH$_3$), 4.12-4.26 (m, 2H, Orn-α-C*H*x2), 4.33-4.43 (m, 1H, Orn-α-C*H*), 5.08 (br s, 2H, C*H*$_2$-Ph), 7.34 (br s, 5H, aromatic); ^{13}C NMR (75 MHz, CD_3OD) δ 13.1 (DIPEA CH$_2$-*C*H$_3$), 18.1 (N-CO*C*H$_3$), 20.3 (DIPEA CH-*C*H$_3$), 24.2, 24.3 (Orn-γ-*C*H$_2$), 30.4, 30.7, 31.0 (Orn-β-*C*H$_2$), 43.6 (DIPEA *C*H$_2$-CH$_3$), 49.8 (Orn-δ-*C*H$_2$, merged with solvent), 54.7 (Orn-α-*C*H), 55.6 (Orn-α-*C*Hx2 and DIPEA *C*H-CH$_3$x2), 67.6 (*C*H$_2$-Ph), 128.8 (*ortho*-C, aromatic), 129.0 (*para*-C, aromatic), 129.5 (*meta*-C, aromatic), 138.1(*ipso*-C, aromatic), 158.3 (*C*ONH Cbz), 173.0, 173.5, 173.7 (*C*ONH), 174.8 (*C*OOH); HRMS for $C_{29}H_{43}N_6O_{12}$ ([M-H]$^-$) calcd: 667.2944 found 667.2946.

Trihydroxamate-based SDC (2.26)

To a solution of **2.18** (35 mg, 0.044 mmol) was added HBTU (20 mg, 0.053 mmol) in DMF (2 mL) and the mixture was stirred for 15 min. In a separate vessel, **2.20** (22 mg, 0.044 mmol) was dissolved in DMF (3 mL). Hereto, TEA (7 μL, 0.053 mmol) was added and both mixtures were combined and stirred at room temperature for 24 h. After completion of reaction, the solvent was evaporated to dryness and the residue thus obtained was dissolved in ethyl acetate and washed with distilled water (25 mLx2). The ethyl acetate layer was collected dried over sodium sulphate. The solvent was evaporated and column chromatography afforded 46 mg (0.036 mmol, 82%) of coupled product **2.22** as a white solid.

^{1}H NMR (300 MHz, CD_3OD) δ 0.88-1.08 (m, 6H, Ile-δ-C*H*$_3$ and Ile-γ-C*H*$_3$), 1.27 (br s, 2H, C*H*$_2$ (C-2)), 1.52-2.52 (m, 33H, Ile-γ-C*H*$_2$, Ile-β-C*H*, Orn-β-C*H*$_2$x3 and Orn-γ-C*H*$_2$x3, N-COC*H*$_3$x3 and N-OCOC*H*$_3$x3), 3.62-3.90 (m, 8H, Orn-δ-C*H*$_2$x3, H-3 and H-5), 4.06 (br s, 2H, H-4, H-6), 4.10-4.22 (m, 3H, H-7a, H-7b and Ile-α-C*H*), 4.30-4.50 (m, 3H, Orn-α-C*H*x3), 4.83 (m, 2H, C*H*$_2$ (C-1), merged with solvent peak), 5.10 (br s, 2H, C*H*$_2$-Ph), 7.35 (br s, 5H, aromatic, Cbz Ph), 7.45-7.55 (m, 3H, aromatic), 8.11 (m, 2H, aromatic); ^{13}C NMR (75 MHz, CD_3OD) δ 11.3 (Ile-δ-*C*H$_3$), 12.2 (Ile-γ-*C*H$_3$), 15.5, 15.8, 15.9 (Orn-γ-*C*H$_2$x3), 20.3 (N-CO*C*H$_3$), 22.1 (N-OCO*C*H$_3$), 25.6 (C-2), 26.0 (Ile-γ-*C*H$_2$), 34.1 (Orn-β-*C*H$_2$x3), 38.1 (Ile-β-*C*H), 51.4 (Orn-δ-*C*H$_2$), 59.9 (C-1), 61.3 (Orn-α-*C*H), 67.8 (Orn-α-*C*H), 69.9 (Orn-α-*C*H), 72.6 (Ile-α-*C*H), 73.0 (C-7), 76.0 (*C*H$_2$-Ph), 80.7 (C-

4), 81.0 (C-3), 82.8 (C-5), 83.5 (C-6), 127.8, 128.7, 128.8, 129.0, 129.5, 130.1, 131.5, 138.2 (aromatic), 158.5 (*C*ONH, Cbz), 166.2 (tetrazole), 170.3, 172.3, 172.8, 173.8, 174.4 (*C*ONH); HRMS for $C_{55}H_{78}N_{12}O_{21}SNa$ ($[M+Na]^+$) calcd: 1297.5017 found 1297.5010.

A solution of coupled product **2.22** (35 mg, 0.027 mmol) in methanolic DIPEA (6%, 10 mL) was stirred at room temperature overnight. After completion of reaction, the solvent was evaporated. The residue was subjected to silica gel chromatography to afford 15 mg (0.013 mmol, 50%) of **2.24** as a yellow oil.

^{1}H NMR (500 MHz, CD_3OD) δ 0.84-0.97 (m, 6H, Ile-γ-C*H*$_3$ and Ile-δ-C*H*$_3$), 1.36 (d, 15H, , *J* = 7.0 Hz, DIPEA C*H*$_3$x5), 1.48-1.96 (m, 13H, Ile-β-C*H*, Ile-γ-C*H*$_2$, (C-2), Orn-β-C*H*$_2$, Orn-γ-C*H*$_2$x3), 2.07 (s, 9H, N-COC*H*$_3$x3), 2.18-2.27 (m, 2H, Orn-β-C*H*$_2$), 2.40-2.50 (m, 2H, Orn-β-C*H*$_2$), 3.21 (q, 2H, *J* = 8.0 Hz, DIPEA C*H*x2,), 3.50-3.75 (m, 8H, Orn-δ-C*H*$_2$x3 and DIPEA C*H*$_2$), 3.75-3.81 (m, 2H, H-3, H-5), 3.82-3.87 (m, 1H, H-4), 4.02 (br s, 1H, H-6), 4.08-4.23 (m, 6H, Orn-α-C*H*x3, Ile-α-C*H*, H-7a, H-7b), 4.36-4.49 (m, 2H, C*H*$_2$-Ph), 7.34 (br s, 5H, aromatic), 7.50 (br s, 3H, aromatic), 8.09 (br s, 2H, aromatic); ^{13}C NMR (125 MHz, CD_3OD) δ 12.1 (DIPEA-CH_2-*C*H_3), 13.2 (Ile-δ-*C*H_3), 16.4 (Ile-γ-*C*H_3), 17.4 (N-CO*C*H_3), 18.8 (Orn-γ-*C*H_2), 20.3 (C-2), 24.2 (DIPEA CH-*C*H_3), 25.8 (Orn-γ-*C*H_2), 30.3 (Orn-β-*C*H_2), 34.2 (Ile-β-*C*H), 43.8 (DIPEA *C*H_2-CH_3), 49.0 (Orn-δ-*C*H_2 merged with solvent peak), 51.4 (C-1), 54.2 (Orn-α-*C*H), 55.8 (DIPEA *C*H-CH_3 and Ile-α-*C*H), 67.8 (*C*H_2-Ph), 69.8 (C-4), 73.1 (C-7), 76.1 (C-3), 80.5 (C-5), 83.7 (C-6), 127.8, 128.8, 128.8, 128.1, 130.1, 131.5, 138.5 (aromatic), 158.8 (*C*ONH, Cbz), 166.1 (tetrazole), 173.8, 179.3 (*C*ONH); HRMS for $C_{49}H_{71}N_{12}O_{18}S$ ($[M-H]^-$) calcd: 1147.4735 found 1147.4702.

To a solution of **2.24** (15 mg, 0.013 mmol) in a mixture of methanol: water (2 mL, 4:1v/v) was added Pd/C (10% w/w, 10 mg) and the reaction mixture was stirred at rt for 5 h under H_2 atmosphere. Subsequently, the reaction mixture was filtered off and washed with the mixture of methanol and water (1:1v/v). The filtrate was collected, the solvent was evaporated under reduced pressure and the residue was purified by HPLC to yield 10 mg (0.01 mmol, 76%) of the desired SDC **2.26** as a white solid.
HRMS for $C_{41}H_{65}N_{12}O_{16}S$ ($[M-H]^-$) calcd: 1013.4360 found 1013.4360.

Trihydroxamat-based SDC (2.27)

The SDC **2.27** was synthesized following the procedure as described for SDC **2.26** with **2.21** substituting for **2.20**. Yield: 20% (overall); HRMS for $C_{55}H_{75}N_{12}O_{19}S$ ($[M-H]^-$) calcd: 1239.4992 found : 1239.4967.

Benzyl-2,3-bis(benzyloxy)-benzoate (2.31)[123]

A solution of **2.30** (2 g, 13.0 mmol), benzyl bromide (9.2 mL, 78.0 mmol) and potassium carbonate (32 g) in acetone (200 mL) was refluxed for 24 h under nitrogen atmosphere. After filtration, the precipitate was washed with acetone. Filtrate and washings were combined, concentrated under vacuum. The residue was subjected to siliga gel chromatography to yield 5.51 g (13.0 mmol, 100%) of **2.31** as a pale yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.06 (s, 2H, C*H*$_2$-Ph), 5.11 (s, 2H, C*H*$_2$-Ph), 5.30 (s, 2H, C*H*$_2$-Ph), 7.02-7.13 (m, 2H, aromatic), 7.22-7.43 (m, 16H, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$) δ 66.9 (*C*H$_2$-Ph), 71.2 (*C*H$_2$-Ph), 75.6 (*C*H$_2$-Ph), 117.9, 122.9, 123.9, 126.8, 126.9, 127.5, 127.8, 128.0, 128.1, 128.1, 128.3, 128.5, 128.5, 135.9, 136.5, 137.3, 148.3, 152.8, 166.1; HRMS for $C_{28}H_{25}O_4$ ([M+H]$^+$) calcd: 425.1747 found 425.1749

2,3-bis(benzyloxy)-benzoic acid (2.32)[123]

A solution of **2.31** (5.51 g, 13.0 mmol) in mixture of methanol (350 mL) and 5N sodium hydroxide (150 mL) was refluxed for 3 h. After 3 h, methanol was evaporated and aqueous part was acidified to pH 2 using 3N HCl and extracted with ethyl acetate (150 mLx4). The organic layer was collected, dried over sodium sulphate, filtered and evaporated to yield 4.08 g (12.21 mmol, 94%) of **2.32** as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.20 (s, 2H, C*H*$_2$-Ph), 5.27 (s, 2H, C*H*$_2$-Ph), 7.15-7.50 (m, 12H, aromatic), 7.75 (m, 1H, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$) δ 65.4 (*C*H$_2$-Ph), 77.2 (*C*H$_2$-Ph), 119.0, 123.0, 124.5, 125.2, 127.0, 127.8, 128.6, 128.8, 128.9, 129.3, 129.3, 134.6, 135.9, 147.1, 151.3, 165.0; HRMS for $C_{21}H_{17}O_4$ ([M-H]$^-$) calcd: 333.1132 found: 333.1109

***N*$^{\alpha}$,*N*$^{\delta}$-bis[(2,3-bis(benzyloxy)-benzoyl)-L-ornithine (2.33)[115]**

To a solution of **2.32** (2 g, 6.0 mmol) and NHS (829 mg, 7.2 mmol) in THF (20 mL) at 0^o C was added a solution of DCC (1.49 g, 7.2 mmol) in THF (10 mL). The reaction mixture was stirred at room temperature for overnight. The resulting DCU was filtered off and washed with THF. The filtrate was used as the active ester solution in the following reaction.

To a solution of **2.8** (1.51 g, 3 mmol) and sodium bicarbonate in water/THF (40 mL/30 mL) at room temperature was added the active ester solution obtained above. The reaction mixture was stirred at room temperature for 6 h. The volatiles were evaporated and aqueous residue was diluted with ethyl acetate and acidified to pH 2 with 1N HCl. The layers were separated and aqueous layer was extracted with ethyl acetate (50 mLx4). The combined extracts were washed with brine, dried over anhydrous sodium sulphate, filtered and concentrated to give crude product which was purified by silica gel column to give 736 mg (0.96 mmol, 32%) of **2.33** as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.18-1.38 (m, 3H, Orn-β-CH_2, Ha and Orn-γ-CH_2), 1.58-1.72 (m, 1H, Orn-β-CH_2, Hb), 2.95-3.07 (m, 1H, Orn-δ-CH_2, Ha), 3.22-3.35 (m, 1H, Orn-δ-CH_2, Hb), 4.60-4.68 (m, 1H, Orn-α-C*H*), 5.03 (s, 2H, CH_2-Ph), 5.10-5.18 (m, 6H, CH_2-Phx3), 7.11-7.16 (m, 4H, aromatic), 7.19-7.24 (m, 4H, aromatic), 7.30-7.48 (m, 15H, aromatic), 7.68-7.74 (m, 2H, aromatic), 8.02 (t, 2H, *J* = 5.7 Hz, NH), 8.53 (d, 1H, *J* = 7.2 Hz, NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.3 (Orn-γ-*C*H_2), 28.7 (Orn-β-*C*H_2), 38.6 (Orn-δ-*C*H_2), 52.0 (Orn-α-*C*H), 71.0 (*C*H_2Ph), 76.0 (*C*H_2-Ph), 117.0, 117.3, 123.0, 123.1, 124.1, 127.3, 127.5, 128.0, 128.2, 128.3, 128.4, 128.5, 128.6, 135.8, 135.9, 136.0, 136.1, 146.6, 146.8, 151.3, 151.4, 165.4, 165.6, (aromatic) 173.4 (*C*=O); HRMS for $C_{47}H_{43}N_2O_8$ ([M-H]$^-$) calcd: 763.3025 found 763.3023.

Biscatecholate-based SDC (2.35)

To a solution of **2.33** (30 mg, 0.04 mmol) in dry DMF (0.5 mL) was added HBTU (18 mg, 0,048 mmol) and the mixture was stirred for 20 min. In a separate vessel, **2.20** (20 mg, 0.04 mmol) and TEA (7 μL, 0.048 mmol) were dissolved in dry DMF (0.5 mL) and both solutions were combined. The resulting reaction mixture was stirred at room temperature overnight. DMF was evaporated and the residue thus obtained was dissolved in ethyl acetate (25 mL) and washed with water (20 mLx2). The aqueous layer was washed with ethyl acetate (20 mLx2). The ethyl acetate layers were combined, dried over anhydrous sodium sulphate, filtered and concentrated to give crude product which was purified by column chromatography to yield 30 mg (0.024 mmol, 61%) of **2.34** as a white solid.

^{1}H NMR (500 MHz, CD_3OD) δ 0.93, (t, 3H, *J* = 6.0 Hz, Ile-δ-CH_3), 1.03 (d, 3H, *J* = 3.0 Hz, Ile-γ-CH_3), 1.23-1.30 (m, 1H, CH_2 (C-2) Ha), 1.40-1.55 (m, 3H, Ile-γ-CH_2, Ha and Orn-γ-CH_2 Ha), 1.58-1.64 (m, 1H, CH_2 (C-2) Hb), 1.72-1.78 (m, 1H, Orn-γ-CH_2 Hb), 1.85-1.91 (m, 1H, Ile-β-C*H*), 2.09-2.15 (m, 1H, Orn-β-CH_2 Ha), 2.31-2.38 (m, 1H, Orn-β-CH_2 Hb), 3.10-3.17 (m, 1H, Orn-δ-CH_2 Ha), 3.22-3.29 (m, 1H, Orn-δ-CH_2 Hb), 3.74-3.78 (m, 2H, H-3, H-5), 3.96-4.03 (m, 2H, H-4 and H-6), 4.19 (d, 1H, *J* = 6.0 Hz, Ile-α-C*H*), 4.31-4.38 (m, 2H, H-7a and H-7b), 4.59-4.64 (m, 1H, Orn-α-C*H*), 4.70-4.80 (m, 2H, CH_2 (C-1)), 5.01-5.07 (m, 3H, CH_2Ph), 5.14-5.20 (m, 5H, CH_2Ph), 7.10-7.29 (m, 29H, aromatic), 8.05-8.10 (m, 2H, aromatic); ^{13}C NMR (125 MHz, CD_3OD) δ 11.5 (Ile-δ-*C*H_3), 15.9 (Ile-γ-*C*H_3), 26.0 (C-2), 26.6 (Ile-γ-*C*H_2), 31.0 (Orn-γ-*C*H_2), 34.1 (Orn-β-*C*H_2), 37.9 (Ile-β-*C*H), 40.1 (Orn-δ-*C*H_2), 51.3 (C-1), 54.2 (Ile-α-*C*H), 60.0 (Orn-α-*C*H), 72.2 (d, *C*H_2Phx2), 72.6 (C-4), 72.9 (C-7), 76.9 (*C*H_2Ph), 77.1 (*C*H_2Ph), 80.9 (C-5), 82.8 (C-6), 118.1, 122.9, 125.5 (d), 127.8, 128.7, 128.9, 129.0, 129.1, 129.2, 129.24, 129.41, 129.44, 129.5, 129.6, 129.9, 130.0, 130.1,131.5, 137.9, 138.0, 138.1, 138.2, 147.6, 147.8, 153.4 (d), 166.1, 167.9 (aromatic), 168.5 (*C*ONH), 174.4 (Ile-*C*=O); HRMS for $C_{67}H_{71}N_8O_{14}S$ ([M-H]$^-$) calcd: 1243.4815 found 1243.4807.

To a solution of coupled product **2.34** (25 mg, 0.02 mmol) in methanol (5 mL) was added Pd/C (10%, 15 mg) and stirred for 4 h under hydrogen atmosphere. After 4 h, the catalyst was filtered off and washed with methanol. The filtrate was concentrated and the residue was purified by RP-HPLC to yield 6.6 mg (0.007 mmol, 37%) of desired biscatecholate based SDC **2.35** as a white solid.

^{1}H NMR (500 MHz, CD_3OD) δ 0.93, (t, 3H, *J* = 4.5 Hz, Ile-δ-C*H*$_3$), 1.02 (d, 3H, *J* = 3.3 Hz, Ile-γ-C*H*$_3$), 1.23-1.33 (m, 1H, C*H*$_2$ (C-2) Ha), 1.57-1.65 (m, 1H, C*H*$_2$ (C-2) Hb), 1.72-1.78 (m, 2H, Ile-γ-C*H*$_2$), 1.83-1.92 (m, 2H, Ile-β-C*H* and Orn-γ-C*H*$_2$ Ha), 1.95-2.02 (m, 1H, Orn-γ-C*H*$_2$ Hb), 2.15-2.23 (m, 1H, Orn-β-C*H*$_2$ Ha), 2.38-2.45 (m, 1H, Orn-β-C*H*$_2$ Hb), 3.38-3.45 (m, 1H, Orn-δ-C*H*$_2$ Ha), 3.46-3.52 (m, 1H, Orn-δ-C*H*$_2$ Hb), 3.77-3.82 (m, 2H, H-3, H-5), 4.02-4.05 (m, 2H, H-4 and H-6), 4.15 (d, 1H, *J* = 7.5 Hz, Ile-α-C*H*), 4.35-4.42 (m, 2H, H-7a and H-7b), 4.67-4.71 (m, 1H, Orn-α-C*H*), 4.82 (2H, C*H*$_2$ (C-1) merged with solvent), 6.67-6.74 (m, 2H, aromatic, catechol), 6.88-6.94 (m, 2H, catechol), 7.21 (d, 1H, *J* = 8.0 Hz, catechol), 7.32 (d, 1H, catechol), 7.46-7.52 (m, 3H, aromatic, tetrazole), 8.07-8.11 (m, 2H, aromatic, aromatic); ^{13}C NMR (125 MHz, CD_3OD) δ 11.3 (Ile-δ-*C*H$_3$), 15.8 (Ile-γ-*C*H$_3$), 26.0 (C-2), 26.9 (Ile-γ-*C*H$_2$), 30.5 (Orn-γ-*C*H$_2$), 34.1 (Orn-β-*C*H$_2$), 37.7 (Ile-β-*C*H), 40.0 (Orn-δ-*C*H$_2$), 51.3 (C-1), 54.2 (Ile-α-*C*H), 60.0 (Orn-α-*C*H), 72.6 (C-4), 73.1 (C-7), 76.0 (C-3), 81.0 (C-5), 82.8 (C-6),116.8, 117.1, 118.8, 119.6, 119.8, 127.8, 128.6, 130.0, 131.5, 147.2, 147.3, 149.6, 150.2, 166.2, 170.9, 171.6, 172.4, 174.9 (aromatic, catechol and aryl-tetrazole); HRMS for $C_{39}H_{47}N_8O_{14}S$ ([M-H]$^-$) calcd: 883.2938 found 883.2933.

(*S*)-*N*$^{\beta}$-Benzyloxycarbonyl-L-α-2,3-diaminopropanoic acid (2.40)[120a, 124]

A slurry of **2.39** (6 g, 22.53 mmol), ethyl acetate (100 mL), acetonitrile (100 mL), water (50 mL) and iodobenzene diacetate (8.73 g, 27.04 mmol) was stirred at 15^o C for min. The temperature was allowed to rise to room temperature and the reaction was stirred for 4.5 h during which the product separated out as a white solid. The reaction mixture was cooled to 5^o C and the product was filtered off and washed with ethyl acetate (200 mL). The precipitate was dried *in vacuo* to afford 4.95 g (20.78 mmol, 92%) of **2.40** as a white solid.

^{1}H NMR (300 MHz, DMSO-d_6/TFA) δ 2.93-3.10 (m, 1H, β-C*H*$_2$-Ha), 3.17-3.30 (m, 1H, β-C*H*$_2$-Hb), 4.21-4.38 (m, 1H, α-CH), 5.05 (s, 2H, C*H*$_2$-Ph), 7.20-7.40 (m, 5H, aromatic), 7.48-7.70 (m, 1H, CON*H*), 7.89 (br s, 2H, N*H*$_2$); ^{13}C NMR (75 MHz, DMSO-d_6/TFA) δ 40.2 (β–*C*H$_2$), 52.6 (α-*C*H), 66.7 (*C*H$_2$-Ph), 110.0, 113.8, 117.6, 121.4 (TFA), 128.6 (Ar), 129.0 (aromatic), 137.4 (*ipso*-C, aromatic), 157.0, 158.5, 159.0, 159.5, 160.0 (TFA), 171.6 (COOH); HRMS for $C_{11}H_{13}N_2O_4$ ([M-H]$^-$) calcd: 237.0881 found 236.0878.

(*S*)-N^{α}-Benzyloxycarbonyl-N^{β}-tert-butyloxycarbonyl--L-α-2,3-diaminopropanoic acid (2.41)[121]

To a solution of **2.40** (1.5 g, 6.3 mmol) in 10% Na_2CO_3 (60 mL) was added a solution of di-tert-butyl dicarbonate (2.1 g, 9.5 mmol) in dioxane (25 mL) over a period of 1h at 0^{o} C. The reaction mixture was stirred at room temperature overnight. The reaction mixture was poured into 100 mL of water after which the mixture was washed with diethyl ether (25 mLx3). The aqueous part was acidified to pH 2 using saturated potassium hydrogen sulphate and the white suspension was extracted with ethyl acetate (50 mLx3). The combined ethyl acetate layer was dried over anhydrous sodium sulphate, filtered and evaporated to dryness. The product was crystallized from diethyl ether to yield 2.06 g (6.09 mmol, 97%) of **2.41** as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.42 (s, 9H, Boc C*H*$_3$), 3.44-3.67 (m, 2H, β-C*H*$_2$), 4.30-4.48 (m, 1H, α-C*H*), 5.03-5.18 (m, 2H, C*H*$_2$-Ph), 6.17 (br s, 1H, CON*H*), 6.42 (br s, 1H, CON*H*), 7.34 (s, 5H, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.4 (Boc *C*H$_3$), 42.4, 43.3 (β-*C*H$_2$), 54.6, 55.7 (α-*C*H), 67.5 (*C*H$_2$-Ph), 128.3 (*para*-C, Ar), 128.4 (*ortho*-C), 128.7 (*meta*-C, Ar), 136.1 (*ipso*-C, aromatic), 155.7, 157.0, 157.4, 158.7 (*C*ONH), 172.9 (*C*OOH); HRMS for $C_{16}H_{21}N_2O_6$ ([M-H]$^-$) calcd: 337.1405 found 337.1382

N^{α}-Benzyloxycarbonyl -L-α-2,3-diaminopropanoyl-N^{α}-Benzyloxycarbonyl-N^{β}-tert-butyloxycarbonyl-L-α-2,3-diaminopropanoic acid (2.42)

To a solution of **2.41** (2 g, 5.91 mmol) and NHS (816 mg, 7.09 mmol) in THF (25 mL) at 0^{o} C was added a solution of DCC (1.46 g, 7.09 mmol) in THF (15 mL). The reaction mixture was stirred at room temperature overnight. The resulting DCU was filtered off and washed with THF. The filtrate was used as active ester solution in the following reaction.

To a solution of **2.40** (1.4 g, 5.91 mmol) and $NaHCO_3$ (1.5 g, 17.73 mmol) in water/THF (40 mL/30 mL) at room temperature was added the active ester obtained from above. The reaction was stirred at room temperature for 6 h. The volatiles were evaporated and the aqueous residue was acidified to pH 2 using saturated $KHSO_4$ and diluted with ethyl acetate (100 mL). The layers were separated and aqueous layer was washed with ethyl acetate (50 mLx3). The combined extracts were washed with brine, dried over anhydrous sodium sulphate, filtered and concentrated. The product was isolated by column chromatography to yield 2.92 g (5.23 mmol, 89%) of **2.42** as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.35 (s, 9H, Boc C*H*$_3$), 3.25-3.50 (m, 2H, β-C*H*$_2$), 3.60-3.80 (m, 2H, β-C*H*$_2$), 4.20-4.45 (m, 2H, α-C*H*x2), 5.08 (br s, 4H, C*H*$_2$-Phx2), 7.30 (br s, 10H, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.3 (Boc *C*H$_3$), 41.2 (β-*C*H$_2$), 42.3 (β-*C*H$_2$), 54.2 (α-*C*H), 56.5 (α-*C*H), 67.3 (*C*H$_2$-Ph), 67.5 (*C*H$_2$-Ph), 80.5 (*C*(CH$_3$)$_3$), 128.4, 128.6 (aromatic), 136.1, 136.2 (*ipso*-C, aromatic), 156.3, 156.9, 157.4 (*C*ONH of Cbz and

Boc), 172.1 (*C*ONH, peptide), 173.1 (*C*OOH); HRMS for $C_{27}H_{33}N_4O_9$ ($[M-H]^-$) calcd: 557.2253 found 557.2254

N^{α}-Benzyloxycarbonyl-L-α-2,3-diaminopropanoyl-N^{α}-Benzyloxycarbonyl- L-α-2,3-diaminopropanoyl-N^{α}-Benzyloxycarbonyl-N^{β}-tert-butyloxycarbonyl-L-α-2,3-diaminopropanoic acid (2.43)[119]

To a solution of **2.42** (558 mg, 1.0 mmol) and NHS (138 mg, 1.2 mmol) in THF (5 mL) at 0^{o} C was added a solution of DCC (248 mg, 1.2 mmol) in THF (5 mL). The reaction mixture was stirred at room temperature overnight. The resulting DCU was filtered off and washed with THF. The filtrate was used as an active ester solution in the following reaction.

To a solution of **2.40** (238 mg, 1.0 mmol) and $NaHCO_3$ (252 mg, 3.0 mmol) in water/THF (5 mL/3 mL) at room temperature was added the active ester obtained from above. The reaction mixture was stirred at room temperature for 6 h. The volatiles were evaporated and aqueous residue was diluted with ethyl acetate (50 mL) and acidified to pH 2 using saturated $KHSO_4$. The layers were separated and aqueous layer was washed with ethyl acetate (50 mLx3). The combined extracts were washed with brine, dried over anhydrous sodium sulphate, filtered and concentrated. The product was isolated by column chromatography to yield 610 mg (0.78 mmol, 78%) of desired tripeptide **2.43** as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (s, 9H, Boc CH_3), 3.37 (br s, 3H, β-CH_2), 3.65 (br s, 3H, β-CH_2), 4.17-4.50 (m, 3H, α-C*H*x3), 4.92-5.13 (m, 6H, CH_2-Phx3), 5.56 (br s, 1H, CON*H*), 6.18 (br s, 1H, CON*H*), 6.51 (br s, 1H, CON*H*), 6.72 (br s, 1H, CON*H*), 7.28 (br s, 15H, aromatic), 7.63 (br s, CON*H*); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.7 (Boc *C*H_3), 42.0, 42.2, 43.0 (β-*C*H_2), 55.3, 56.2, 57.3 (α-*C*H), 67.8, 68.0 (*C*H_2-Ph), 80.7 (*C*$(CH_3)_3$), 128.9, 129.0, 129.5 (aromatic), 137.9, 138.0, 138.1 (*ipso*-C, aromatic), 158.3, 158.6, 158.8, 172.9, 172.9 (*C*ONH), 173.5 (*C*OOH); HRMS for $C_{38}H_{45}N_6O_{12}$ ($[M+Na]^+$) calcd: 777.3101 found 777.3087

L-α-2,3-diaminopropanoyl-L-α-2,3-diaminopropanoyl-N^{β}-tert-butyloxycarbonyl-L-α-2,3-diaminopropanoic acid (2.44)

To a solution of **2.43** (110 mg, 0.14 mmol) in a mixture of methanol: water (4:1 v/v, 5 mL) was added 30 mg of Pd/C (10% w/w, 30 mg) and the mixture was stirred under hydrogen atmosphere for 6h. After 6h, the catalyst was filtered off and the solvent was evaporated to yield 48 mg (0.12 mmol, 91%) of **2.44** which was used without further purification.

^{1}H NMR (300 MHz, D_2O) δ 1.44 (s, 9H, Boc-CH_3), 3.35-3.45 (m, 2H, β-CH_2), 3.55-3.80 (m, 6H, β-CH_2x2 and α-C*H*x2), 3.85-3.92 (m, 1H, α-C*H*); ^{13}C NMR (75 MHz, D_2O) δ

27.3 (Boc-*C*H_3), 39.5, 39.6, 42.1 (β-*C*H_2), 53.2, 53.6, 54.6 (α-*C*H), 81.0 (*C*$(CH_3)_3$); HRMS for $C_{14}H_{27}N_6O_6$ ([M-H]$^-$) calcd: 375.1997 found 375.1989.

Methyl-(L-α-2,3-diaminopropanoyl-L-α-2,3-diaminopropanoyl-N^{β}-tert-butyloxycarbonyl)--L-α-2,3-diaminopropanoate (2.46)[119]

To a solution of **2.44** (100 mg, 0.13 mmol) in a mixture of dry DCM (5 mL) and dry DMF (0.5 mL) were added HBTU (61 mg, 0.16 mmol), methanol (1 mL) and TEA (22 μL, 0.16 mmol). The reaction mixture was stirred at room temperature for 2 h. Next, the volatiles were removed and the residue was partitioned between ethyl acetate and 2N HCl. The aqueous part was extracted with ethyl acetate (25 mLx2). The combined extracts were dried over anhydrous sodium sulphate, filtered and evaporated to dryness. The residue was subjected to silica gel purification to yield 98 mg (0.12 mmol, 96%) of the intermediate Cbz protected tripeptide methyl ester as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.31 (s, 9H, Boc-CH_3), 3.20-3.90 (m+s, 9H, β-CH_2x3 and COOCH_3), 4.10-4.50 (m, 3H, α-C*H*), 5.06 (br s, 6H, CH_2Phx3), 6.26 (br s, 2H, CON*H*x2), 6.69 (br s, CON*H*, 7.09 (br s, 1H, CON*H*), 7.31 (br s, 15H, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$), 28.3 (Boc-*C*H_3), 41.4, 41.8, 42.3 (β-*C*H_2), 53.0 (COO*C*H_3), 54.2, 56.0, 56.1 (α-*C*H), 67.2, 67.3, 67.4 (*C*H_2Ph), 80.7 (*C*$(CH_3)_3$), 128.2, 128.3, 128.59, 128.64 (aromatic), 136.1, 136.2, 136.3 (ipso-C, aromatic), 156.5, 156.6 (*C*ONH), 171.2, 171.8, 172.1 (*C*ONH and *C*OCH_3); MS for $C_{39}H_{48}N_6O_{12}Na$ ([M+Na]$^+$) calcd: 815.3 found 815.1.

To a solution of this tripeptide methyl ester above (56 mg, 0.07 mmol) in THF: acetic acid (3:2 v/v, 5 mL) was added Pd/C (10%w/w, 50 mg) and the mixture was stirred at room temperature under H_2 atmosphere for 18 h. Next, the catalyst was filtered off and the solvent was evaporated to dryness to yield 23 mg (0.04 mmol, 57%) of **2.46** which was used further without purification. However all attempts to couple catechole **2.32** with this tripeptide failed.

2.5.2 Biological activity of SDCs

2.5.2.1 Whole-cell assay by disc diffusion method

Disc diffusion method using Muller-Hinton Agar (MHA)

Liquid Mueller–Hinton Agar (0.75%) was mixed with bacterial suspension (29:1 v/v) at rt. Plates were prepared by adding 4 mL of this suspension to petri plates containing 7 mL of solidified Mueller–Hinton Agar (1.5%). Immediately after solidification of the agar, 2 μL drops containing potential inhibitor compound in milliQ water were added and were allowed to dry. After incubation for 16 h at 37^o C, the inhibition zones were measured. These evaluations were performed in triplicate for each compound-concentration combination. Maximum concentration tested was 5 mM.

Disc diffusion method using M63

M63 soft Agar (0.75% of agar) was mixed with bacterial suspension (29:1 v/v) at rt. Plates were prepared by adding 4 mL of this suspension to petri plates containing 7 mL of solidified M63 hard Agar (1.5% of agar). Immediately after solidification of the agar, 2 µL drops containing potential inhibitor compound in milliQ water were added and were allowed to dry. After incubation for 16 h at 37^{o} C, the inhibition zones were measured. These evaluations were performed in triplicate for each compound-concentration combination. Maximum concentration tested was 5 mM

2.5.2.2 MIC determination by broth dilution method

The respective bacteria were grown overnight in LB medium and cultured again the following day in fresh LB medium to an OD600 of 0.1. Compounds were titrated in a 96 well-plate using LB-medium with or without iron chelator such as 2,2'-dipyridyl or EDTA (final concentration of 10 µg/mL), and with or without $FeCl_3$ (equimolar with SDC) which was used as iron source to promote the uptake of SDC. To each well 85 µL LB-medium with or without iron chelator and iron source was added to a total volume of 90 µL followed by 10 µL of bacterial cell culture. The cultures were next incubated at 37 °C and the OD600 was determined at two intervals after 8 h and after 18 h respectively.

2.5.2.3 Aminoacylation experiments

To assess the degree of inhibition of the aminoacylation reaction, *in vitro* tests were performed using the relevant S30 cell extracts.

Preparation of S30 cell extracts: The cell extracts were prepared as described as per literature procedure.[78] The respective *E. coli* cells were grown overnight at 37^{o} C in LB medium (5 mL). Next day, the overnight grown cell culture (100 µL) was transferred to 200 mL LB medium and allowed to attain OD_{600} of 0.8-1.0. Next the cell culture was centrifuged at 3000xg for 10 min. The supernatant was discarded and the pellet was resuspended in 40 mL buffer containing: Tris.HCl or Hepes.KOH (pH = 8.0) (20 mM), $MgCl_2$ (10 mM), KCl (100 mM). The cell-suspension was centrifuged again at 4800 rpm for 15 min. This procedure was repeated 3 times. The pellet was resuspended in 1 mL of the following buffer Tris.HCl or Hepes.KOH (pH = 8.0) (20 mM), $MgCl_2$ (10 mM), KCl (100 mM), DTT (1 mM) and kept at 0 °C. Subsequently, the cells were sonicated for 30 sec. and left at 0°C for 10 sec. This procedure was repeated for 15-20 times. The cell lysate was centrifuged to a maximum speed of 15000 g for 30 min. The supernatant was transferred and centrifuge again for 15 min. The supernatant was transferred and divided over several eppendorf tubes (60 µL each) and the extract was stored at -80 ^{o}C until further use.

tRNA aminoacylation reaction: To 1 μL of solution containing inhibitor, 3 μL of *E. coli* S30 extract was added. Next, 16 μL of the following aminoacylation mixture was added: Tris.HCl (30mM, pH 8.0), DTT (1 mM), bulk of *E. coli* tRNA (5 g/l), ATP (3 mM), KCl (30 mM), $MgCl_2$ (8 mM), and the specified, radio labeled amino acid (40 μM). The aminoacylation reaction was carried out at room temperature. Depending on whether or not processing was needed, variable time intervals were included between the addition of the cell-extract and the addition of the aminoacylation mixture. The reaction products were precipitated in cold 10% TCA on Whatman 3MM papers, 5 min. after the aminoacylation mixture was added.After thorough washing with cold 10% TCA, the papers were washed twice with acetone and dried on a heating plate. Following the addition of scintillation liquid, the amount of radioactivity was determined in a scintillation counter.

3 Base substituted 5'-*O*-(*N*-isoleucyl)sulfamoyl nucleoside analogues as potential antibacterial agents

Abstract

Aminoacyl-sulfamoyl adenosines are well-known nanomolar inhibitors of the corresponding prokaryotic and eukaryotic tRNA synthetases *in vitro*. Inspired by the aryl-tetrazole containing compounds of Cubist Pharmaceuticals and the modified base as found in the natural antibiotic albomycin, the selectivity issue of the sulfamoylated adenosines prompted us to investigate the pharmacophoric importance of the adenine base. We therefore synthesized and evaluated several isoleucyl-sulfamoyl nucleoside analogues with either uracil, cytosine, hypoxanthine, guanine, 1,3-dideaza-adenine (benzimidazole) or 4-nitro-benzimidazole as the heterocyclic base. Based on the structure and antibacterial activity of microcin C, we also prepared their hexapeptidyl conjugates in an effort to improve their uptake potential. We further compared their antibacterial activity with the parent isoleucyl-sulfamoyl adenosine (Ile-SA), both in *in vitro* and in cellular assays. Surprisingly, the strongest *in vitro* inhibition was found for the uracil containing analogue **3.11f**. Unfortunately, only very weak growth inhibitory properties were found as of low uptake. The results are discussed in the light of previous literature findings.

This chapter was published earlier in an adapted form as:

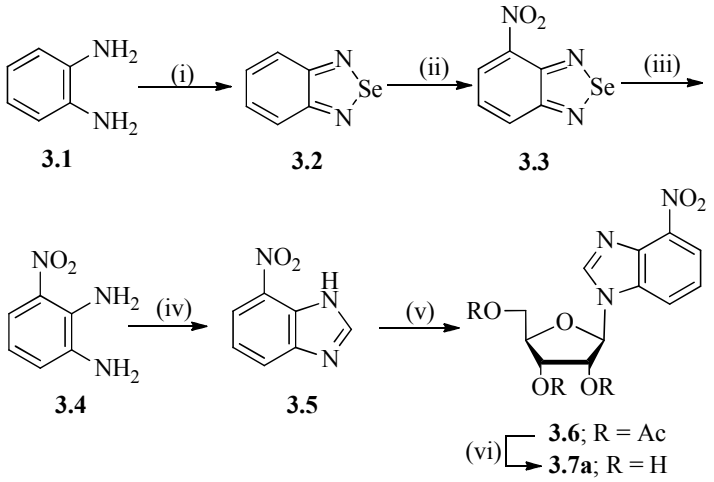
"Base substituted 5'-*O*-(*N*-isoleucyl)sulfamoyl nucleoside analogues as potential antibacterial agents." B. Gadakh, G. Vondenhoff, E. Lescrinier, J. Rozenski, M. Froeyen and A. Van Aerschot, *Bioorg. Med. Chem*, **2014**, 22(10), p-2875-2886

3.1 Introduction

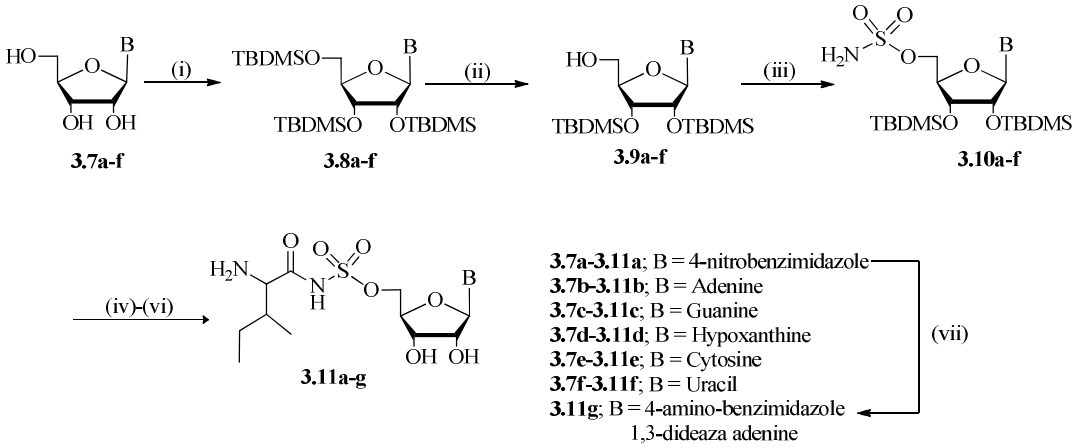
5'-*O*-(*N*-aminoacyl)-sulfamoyl adenosines are well-known inhibitors of the corresponding aaRSs *in vitro* but lack selectivity due to their structural similarity with aa-AMP. Several modifications have been attempted in order to increase the selectivity of these analogues. For instance, Cubist Pharmaceuticals reported aryl-tetrazole containing sulfamate derivatives which displayed up to 3000-fold selectivity for bacterial aaRS over their human counterpart. However, further development was halted due to their lack of *in vivo* activity and high serum albumin binding.[8, 61] Moreover, the natural antibiotic such as albomycin has a modified pyrimidine base and mupirocin has no base moiety at all. Thus, high selectivity of aryl-tetrazole containing sulfamates[8, 61] and the excellent efficacy of albomycin against *Streptococcus pneumonia,*[96] both deviating from the adenine base structure while yet acting as aa-AMP analogues, prompted us to investigate the pharmacophoric importance of the adenine base for the well-known aminoacyl sulfamoyl adenosine (aaSA) inhibitors. We therefore intended to evaluate a series of aaSA analogues with different natural or unnatural heterocyclic bases substituting for the adenine ring. Towards this end, we designed, synthesized, and evaluated several isoleucyl-sulfamoyl nucleoside analogues with either uracil (U), cytosine (C), hypoxantine (I), guanine (G), 1,3-dideaza-adenine [4-amino-benzimidazole (4-ABI)] or 4-nitro-benzimidazole (4-NBI) as the heterocyclic base. We further compared their antibacterial activity with the parent Ile-SA, both *in vitro* and in cellular assays.

3.2 Chemistry

The corresponding series of isoleucyl sulfamate analogues were prepared combining different literature procedures. The required nitrobenzimidazole heterocycle was synthesized starting from *o*-phenylene diamine **3.1** via its selenium complex **3.2**[125] followed by nitration,[126] reduction,[127] and cyclization[128] using triethyl orthoformate yielding 4(7)-nitrobenzimidazole **3.5** (Scheme 3-1). The heterocyclic base was further reacted with tetra-*O*-acetyl ribofuranose using stannic chloride[128] followed by deprotection of the acetyl moieties using methanolic ammonia yielding the 4-nitro benzimidazole nucleoside analogue **3.7a**. All sulfamoylated nucleoside analogues (natural and unnatural ones) were further synthesized following literature procedures which



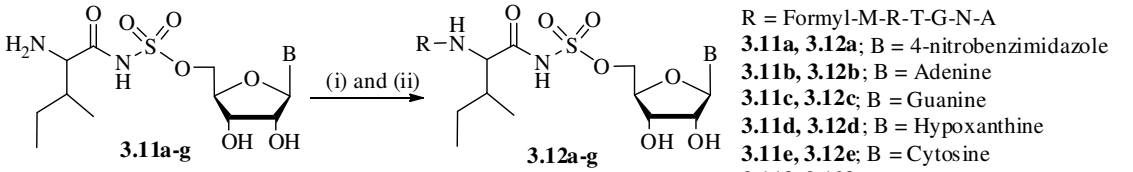
Scheme 3-1: Synthesis of 1-(β-D-ribofuranosyl)-4-nitrobenzimidazole
Reagents and conditions: (i) ethanol, selenium dioxide, reflux 10 min, 99%; (ii) conc. H_2SO_4 and conc. HNO_3 from 0° C to rt in 30 min, 100%; (iii) conc. HCl, aq. HI (57%), 3 h, 54%; (iv) triethyl orthoformate, reflux, 4 h then formic acid relux 3 h, 87%; (v) tetra-*O*-acetyl ribofuranose, $SnCl_4$ (1M solution in DCM), dry ACN, 25° C, 16 h, 80%; (vi) 7N ammonia in methanol, rt, overnight, 90%.



Scheme 3-2: Synthetic scheme for assembly of 5'-*O*-(*N*-isoleucyl)-sulfamoyl adenosine and its different base analogues (**3.11a-g**).
Reagents and conditions: (i) TBDMSCl, imidazole, dry DMF, 50 °C, 3 d, 92-98%; (ii) TFA-Water, THF, 0 °C, 6 h, 90-99%; (iii) $ClSO_2NCO$, formic acid, DMA, 63-80%; (iv) Boc-Ile-OSu, DBU, DMF, rt, 6 h, (v) TFA:water (5:2), rt, 3 h, (vi) TEA.3HF, THF, rt, overnight 28-65% (over 3 steps); (vii) Pd/C, methanol: water, H_2 atm, rt, 6 h, 40%.

involved persilylation of the nucleoside followed by selective removal of the 5'-TBDMS protection and reaction of the liberated 5'-hydroxyl group with in situ formed sulfamoyl

chloride.[82] Finally, the isoleucyl sulfamate analogues were obtained by coupling of the active ester of Boc-Ile (in form of Boc-Ile-OSu) with the respective sulfamoylated analogues (**3.10a-f**) using DBU as a base in DMF.The Boc group was deprotected by acidolysis using TFA/water and TBDMS groups were cleave by $Et_3N.3HF$ affording the desired isoleucyl sulfamate analogues (**3.11a-f**). Finally, the 4-nitro moiety in **3.10a** was reduced by hydrogenation using Pd/C under H_2 atmosphere to yield **3.11g** (Scheme 3-2). The hexapeptide uptake signal was further coupled to the analogues **3.11a-g** to afford derivatives **3.12a-g** following a previous protocol [81] (Scheme 3-3).



Scheme 3-3: Strategy for synthesis of the different nucleoside sulfamate-hexapeptidyl conjugates (**3.12a-g**).
Reagents and conditions: (i) DIC, HOBt, DIPEA, DMF, 16 h, (ii) TFA/water/thioanisole (90:7.5:2.5), rt, 2 h, 6-12 % (over 2 steps, after HPLC purification)

3.3 Biological evaluation

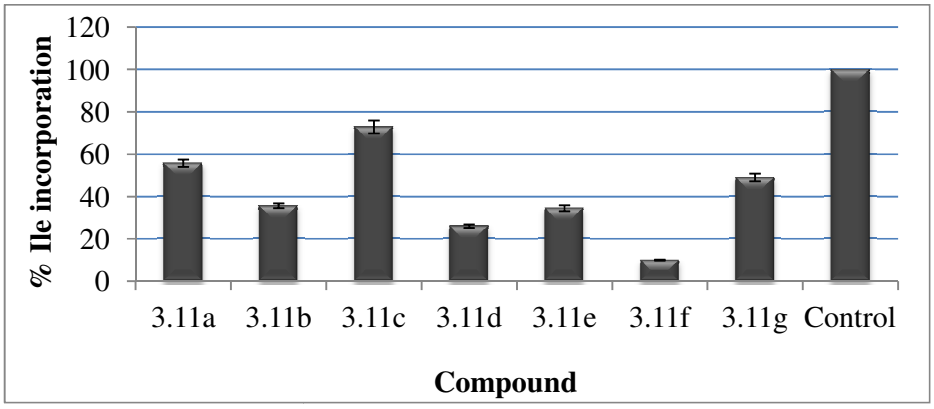


Figure 3-1: *In vitro* aminoacylation reaction in presence of the respective aaSA analogues **3.11a-g** using *E. coli* wt S30 cell extracts at 156 nM final concentration.

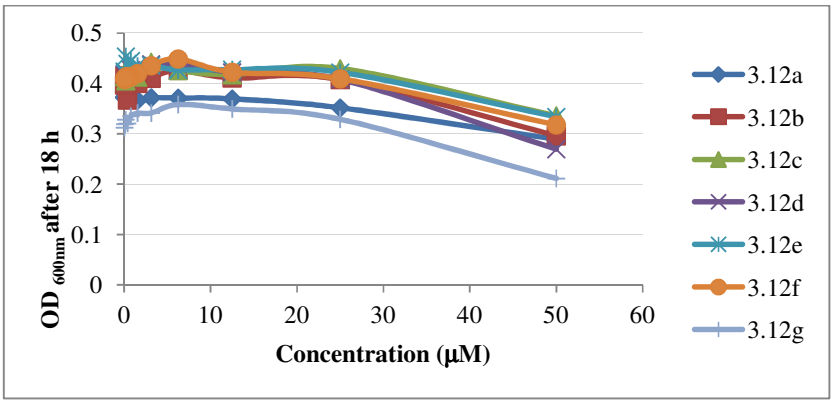
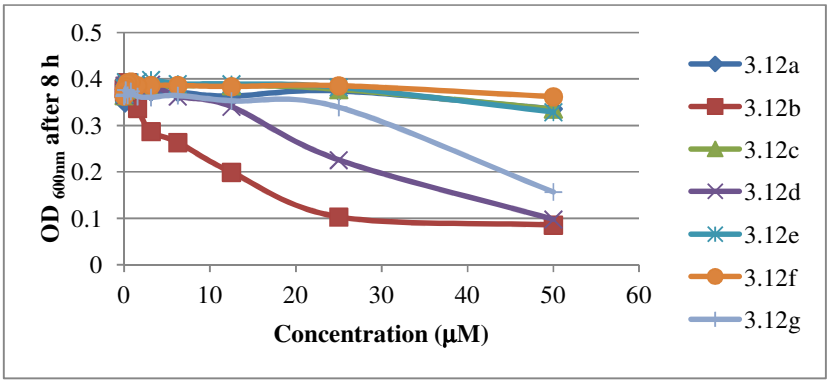


Figure 3-2: Broth dilution antibacterial activity test against *E. coli* wt in LB medium at different concentrations of the hexapeptidyl conjugates **3.12a-g.**

A)



B)

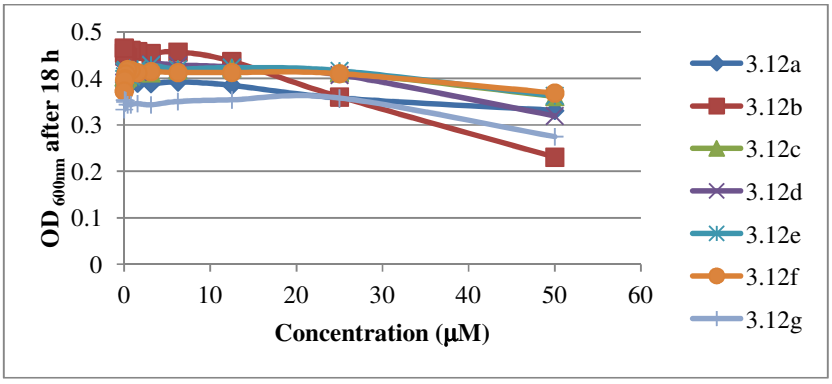


Figure 3-3: Broth dilution antibacterial test against *E. coli* Ara-Yej (BW39758) strain in LB medium containing 5 mM L-arabinose at different concentrations of the hexapeptidyl conjugates **3.12a-g** as determined after incubation for 8 h (panel A) and after 18 h (panel B).

The obtained analogues were evaluated for inhibition of isoleucine incorporation under *in vitro* aminoacylation experiment (Figure 3-1). The remarkable order of inhibitory activity found for the different sulfamates surprisingly showed the strongest inhibition for the U analogue **3.11f** followed by I > adenine= C > 4-ABI > 4-NBI > G. Excited with the results from our *in vitro* aminoacylation experiments, we evaluated the analogues **3.11a-g** for their ability to inhibit the growth of microorganisms by a disc diffusion method (data not shown).

In line with previous results[81] none of the analogues showed growth inhibitory activity (maximum concentration tested was 5 mM). Hexapeptidyl (formyl-MRTGNA-OH) conjugates of these analogues likewise were evaluated for their growth inhibitory properties. Unfortunately, none of conjugates **3.12a-g** displayed any activity against *E. coli* wt (Figure 3-2). Upon evaluation with the *E. coli* Ara-Yej inducer strain (BW39758)[81] transient inhibition could be seen after 8 h incubation for the adenosine conjugate **3.12b** and to a lower extent for the purines **3.12d** and **3.12g**. The uracil conjugate **3.12f** however was devoid of inhibitory activity (panel A, Figure 3-3).

3.4 Discussion

Several non-hydrolysable analogues of aa-AMP have been reported in literature as inhibitors of aaRS with the aaSAs displaying nanomolar affinity for the corresponding aaRS *in vitro*. These inhibitors however lack selectivity for bacterial aaRSs as compared to their human orthologs due to structural similarity with aa-AMP. Visual inspection of the compounds reported by Cubist Pharmaceuticals, and of albomycin and mupirocin reveals that these structures vary from aaSA analogues in having a heterocyclic base or a modified base or no base moiety at all as in mupirocin respectively. These observations prompted us to re-investigate the pharmacophoric importance of the adenine base in aaSAs. IleRS, being extensively studied as aaRS target before (e.g. the mupirocin studies and the Cubist Pharmaceuticals inhibitor program), was chosen to study the effect of different bases substituting for adenine. Hereto, isoleucyl-sulfamoyl nucleoside analogues comprising either a natural base like G, I, U or C, or the unnatural base 4-NBI or 4-ABI (the latter corresponding to 1,3-dideaza adenine) were envisaged. The natural nucleobase containing analogues were prepared to investigate the effect of pyrimidine versus purine analogues, whereas the sulfamates containing an unnatural base aimed at evaluating the

importance of N^1 and N^3 of the adenine ring, respectively. All analogues were synthesized as outlined in Scheme 3-2.

From the *in vitro* aminoacylation experiments it can be concluded that these molecules act as IleRS inhibitors (Figure 3-1). The remarkable order of inhibitory activity found for the different sulfamates showed the strongest inhibition for the U analogue **3.11f** followed by I > adenine= C > 4-ABI > 4-NBI > G. This implies that the adenine base or a purine ring per se is not a prerequisite for aaRS inhibition. Furthermore, U and C analogues proved to be either more or equally active respectively, as compared to the adenine base. The latter is rather unexpected in view of their small size occupying only part of the active site. While I is well tolerated, addition of the C-2 amino moiety as in the G analogue resulted in a significant decrease in activity possibly due to steric clashes. The unnatural 1,3-dideaza-adenine containing analogues displayed lower activity, most probably in view of loss of the reported H-bonding of the adenine N3 position with a His residue of the active site.[129] The 4-NBI analogue **3.11a** proved less active compared to its reduced counterpart, the 4-ABI (1,3-dideaza adenine) analogue suggesting the possible interaction of the amine with the synthetase active site. In contrast, in the reported IleRS.IleAMS of Nakama *et al.* (with IleAMS being a close analogue to IleSA with nitrogen substituting for the 5'-oxygen), the adenine N6 atom is too far to allow hydrogen bonding.[129]

The strong *in vitro* inhibition shown by both pyrimidine containing compounds is rather unexpected in view of the omnipresent and more spacious adenosine being used in the majority of biochemical processes. We therefore tried to get some insight into the binding pocket of IleRS, and made an attempt to carry out some molecular simulations with the synthesized inhibitors using a homology modeled IleRS. The *E. coli* IleRS structure being not available in the protein databank, a homology model was created using the I-tasser server[130] starting from the *E. coli* MetRS structure (1PG0); (see experimental section). The newly developed inhibitors were then superimposed onto the original inhibitor present in 1PG0 (Figure 3-4).

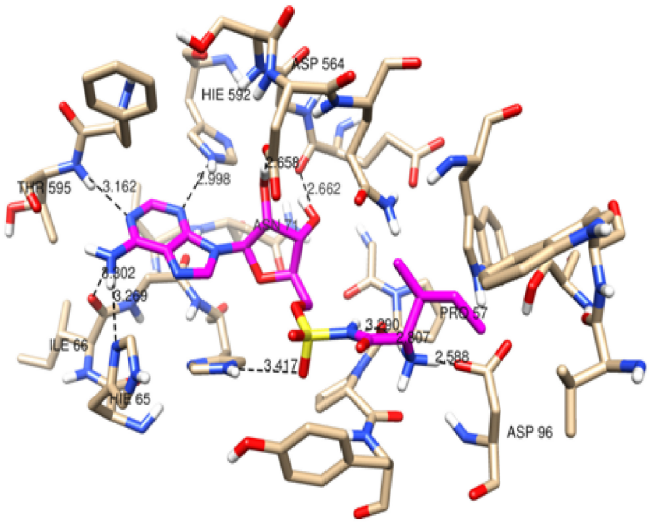


Figure 3-4: Homology model for *E. coli* IleRS based on the 1PG0 structure including its interactions with the sulfamoylated adenosine analogue **3.11b**.

In using a homology model for our analysis, we cannot use a classical program to dock the inhibitors into the binding site of tRNA synthetase. Numerous amino acid side chains, in addition to the many flexible dihedrals from the inhibitors, would have to be adjusted to accommodate the inhibitors. Unfortunately, the noise on the calculation of the interaction energy values for the different inhibitors proved too large to allow for a clear classification.

Despite the exciting results from the *in vitro* aminoacylation experiments, no growth inhibitory activity on microorganisms was found for the analogues **3.11a-g** by a disc diffusion method up to maximum concentration tested of 5 mM. This lack of activity corresponds with the general lack of activity in a whole-cell assay as shown before[81] and can be related to limited cell-penetration in accordance with the lack of *in vivo* efficacy for the IleSA analogue **3.11b**. Hence with the microcin C strategy in mind, hexapeptidyl conjugates of these analogues were prepared in an effort to improve the uptake of these compounds via a Trojan-horse mechanism. Hence, the conjugates **3.12a-g** were evaluated against *E. coli* wt (Figure 3-2) or *E. coli* Ara-Yej inducer (BW39758) strain (Figure 3-3) in a broth dilution test. The latter strain upon arabinose induction shows an increased expression of the transporter.[81] Unfortunately, only some transient antibacterial activity was noticed for purine derivatives **3.12b**, **3.12d** and **3.12g** following incubation with the inducer strain (panel A, figure 3-3), while no antibacterial activity could be recorded for

the *in vitro* strongly active uracil derivative **3.12f**. As it has been shown previously that the hexapeptide is effectively metabolized by non-specific peptidases failure of uptake is the likely reason for the low activity of these hexapeptidyl conjugates. Hence, being the only different part of these constructs, the heterocyclic base might play an important role for recognition by the transporter as was seen with the tetrazole analogues when coupled to the peptidic carrier.[83]

3.5 Conclusions

A series of isoleucyl-sulfamoylated nucleoside analogues comprising different base substitution was synthesized in an effort to evaluate the pharmacophoric importance of the adenine ring for recognition by IleRS. Upon *in vitro* evaluation in a cellular extract, surprisingly the uracil containing analogue **3.11f** was shown to be endowed with the highest inhibitory properties. To promote their bacterial uptake, these polar sulfamoylated analogues were converted into their respective hexapeptidyl conjugates in analogy with the natural Trojan-horse antibiotic microcin C. Unfortunately, only weak inhibitory properties could be noticed for the adenosine analogue **3.12b** and not for the conjugate **3.12f** comprising the uracil moiety.

3.6 Experimental section

3.6.1 Materials and Methods:

Analogous to the procedure as described in section 2.5.1 In addition, signal assignment for ^{1}H and ^{13}C in the conjugate compounds **3.12a-g** were obtained using [^{1}H,^{1}H]-DQF-COSY,[131] [^{1}H,^{13}C]-HSQC[132] and [^{1}H,^{13}C]-HMBC[133] spectra were recorderd on Bruker Avance II 600 with a TCI gradient cryoprobe.

2,1,3-benzoselenadiazole (3.2)
An amount of **3.1** (10.5 g, 97.1 mmol) and selenium dioxide (11.85 g, 106.8 mmol) were refluxed in absolute ethanol (100 mL) for 10 min. The reaction mixture was cooled to rt and ethanol was evaporated *in vacuo*. The product was precipitated from water to yield 17.6 g (96.1 mmol, 99% yield) of **3.2** as a faint pink coloured solid: ^{1}H NMR (300 MHz, CD_3OD) δ 7.46-7.52 (m, 2H, Ar), 7.76-7.83 (m, 2H, Ar); ^{13}C NMR (75 MHz, CD_3OD) δ 124.2, 130.6, 161.6 (C=N); HRMS for $C_6H_5N_2Se$ ($[M+H]^+$) calcd: 184.9612 found 184.9616.

4-nitro-2,1,3-benzoselenadiazole (3.3)[126]

A mixture of 9.1 mL of conc. nitric acid and 18.2 mL of conc. sulphuric acid was added to a solution of **3.2** (16.7 g, 91.2 mmol) in conc. sulphuric acid (36.5 mL) at 0 ^{o}C. The solution was allowed to stir at room temperature for 30 min and then poured into excess ice water. The precipitate was filtered off and washed with distilled water to yield 20.8 g (91.2 mmol, quantitative) of the title compound **3.3** as yellow solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.72-7.78 (dd, 1H, J = 7.5 Hz and 8.7 Hz, H-*meta* to nitro), 8.29 (d, 1H, J = 8.7 Hz, H-*para* to nitro), 8.46 (d, 1H, J = 7.5 Hz, H-*ortho* to nitro); ^{13}C NMR (75 MHz, DMSO-d_6) δ 126.4, 126.8, 129.4, 140.5, 149.9, 159.9; HRMS for $C_6H_4N_3O_2Se$ ($[M+H]^+$) calcd: 229.9463 found 229.9668.

3-nitro-1,2-phenylenediamine (3.4)[128]

To a suspension of **3.3** (18 g, 79 mmol) in conc. hydrochloric acid (225 mL), 70 mL of aqueous hydroiodic acid (57% w/v, 70 mL) was added dropwise at room temperature with vigorous stirring. The reaction mixture was stirred further at room temperature for 3 h. A 5% aqueous sodium hydrogen sulphide solution (400 mL) was added to the dark-red reaction mixture. This was then warmed to 80 ^{o}C and filtered hot and afterwards cooled to 4 ^{o}C. The needle like salt of the diamine crystallized out. The product was neutralized with 30% NaOH to pH 8 and extracted with ethyl acetate (6x100 mL). The ethyl acetate layer was collected, dried over Na_2SO_4, filtered off and evaporated to yield 6.5 g (42.44 mmol, 54%) of the title compound **3.4** as a red solid: ^{1}H NMR (300 MHz, CD_3OD) δ 6.47-6.56 (dd, 1H, J = 7.5 Hz and 8.7 Hz, Ar), 6.87-6.92 (dd, 1H, J = 1.2 Hz and 7.5 Hz, Ar), 7.48-7.53 (dd, 1H, J = 1.2 Hz and 8.7 Hz, Ar); ^{13}C NMR (75 MHz, CD_3OD) δ 116.4, 117.0, 120.6 (quaternary carbon not detected); HRMS for $C_6H_7N_3O_2Na$ ($[M+Na]^+$) calcd: 176.0431 found 176.1058.

4(7)-nitro-benzimidazole (3.5)[128]

A mixture of **3.4** (6 g, 39.2 mmol) and triethyl orthoformate (150 mL) was refluxed at 145 ^{o}C for 4 h. The solution was evaporated to dryness with a rotary evaporator and the residue obtained was dissolved in formic acid (150 mL) and refluxed for 3 h at 110 ^{o}C. When the reaction was completed, the excess formic acid was removed *in vacuo* and the residue was dissolved in methanol (150 mL) and treated with activated charcoal by overnight stirring at room temperature. The charcoal was removed by filtration through celite and the filtrate was evaporated to dryness. The residue obtained was subjected to silica gel column chromatography to afford 5.54 g (34 mmol, 87 %) of **3.5** as a yellow solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.43 (t, 1H, J = 8.1 Hz, H-5/6), 8.16 (d, 2H, J = 8.1 Hz, H-4/7 and H-6/5), 8.45 (s, 1H, H-2), 8.47 (bs, 1H, N*H*); ^{13}C NMR (75 MHz, DMSO-d_6) δ 118.9 (C-5/6), 121.2 (C-4/7), 126.6 (C-6/5), 145.3 (C-2); HRMS for $C_7H_4N_3O_2$ ($[M-H]^-$) calcd: 162.0309 found 162.0314.

1-(2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl)-4-nitrobenzimidazole (3.6)[128]

The compound **3.5** (1.2 g, 7.36 mmol) and 1',2',3',5'-tetra-*O*-acetyl-β-D-ribofuranose (2.81 g, 8.83 mmol) were dissolved in 30 mL of dry acetonitrile. A solution of stannic chloride (1M in DCM, 22.1 mL, 22.1 mmol) was added and the reaction mixture was stirred at 25 ^{o}C for 16 h. The reaction mixture was diluted with DCM and then poured under stirring into 30 mL of ice cooled saturated sodium bicarbonate. The resulting suspension was filtered through celite and the layers were separated. The organic layer was further washed with brine and was dried over Na_2SO_4, filtered off and evaporated. The residue was purified by silica gel column chromatography to yield 2.5 g (5.93 mmol, 80%) of the title compound **3.6** as pale yellow solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.10 (s, 3H, COC*H*$_3$), 2.16 (s, 3H, COC*H*$_3$), 2.18 (s, 3H, COC*H*$_3$), 4.39-4.44 (m, 1H, H-5'a), 4.45-4.50 (m, 1H, H-5'b), 4.51-4.55 (m, 1H, H-4'), 5.51 (t, 1H, *J* = 5.0 Hz, H-3'), 5.56 (t, 1H, 5.5 Hz, H-2'), 6.15 (d, 1H, *J* = 5.5 Hz, H-1'), 7.44 (t, 1H, *J* = 8.0 Hz, H-6), 7.97 (d, 1H, *J* = 8.0 Hz, H-7), 8.19 (d, 1H, *J* = 8.0 Hz, H-5), 8.40 (s, 1H, H-2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 19.5 (CO*C*H$_3$), 19.6 (CO*C*H$_3$), 19.9 (CO*C*H$_3$), 61.7 (C-5'), 69.1 (C-3'), 72.5 (C-2'), 79.7 (C-4'), 86.5 (C-1'), 116.3 (C-5), 119.2 (C-7), 122.1 (C-6), 134.2 (C-9), 136.9 (C-4), 138.8 (C-8), 142.8 (C-2), 168.5 (*C*OCH$_3$), 168.7(*C*OCH$_3$), 169.1 (*C*OCH$_3$); HRMS for $C_{18}H_{19}N_3O_9Na$ ($[M+Na]^+$) calcd: 444.1014 found 444.1012.

1-β-D-(4-nitro-benzimidazol-1-yl)-ribofuranoside (3.7a)

The acylated precursor **3.6** (2.5 g, 5.93 mmol) was dissolved in methanolic ammonia (7N, 25 mL) at 0 ^{o}C. The reaction mixture was allowed to warm to room temperature and stirred overnight. Then the mixture was evaporated to dryness and the residue was partitioned between water and ether. The aqueous layer was evaporated to yield 1.4 g (4.74 mmol, 90%) of the title compound **3.7a** as a pale yellow solid which was used further without purification: ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 3.62-3.66 (m, 1H, H-5'a), 3.67-3.72 (m, 1H, H-5'b), 4.03 (q, 1H, *J* = 3.0 Hz, H-4'), 4.14 (q, 1H, *J* = 5.0 Hz, H-3'), 4.38 (q, 1H, *J* = 6.0 Hz, H-2'), 5.19 (t, 1H, *J* = 5.5 Hz, 5'-OH), 5.27 (d, 1H, *J* = 4.5 Hz, 3'-OH), 5.56 (d, 1H, *J* = 6.0 Hz, H-1'), 7.48 (t, 1H, *J* = 8.0 Hz, H-6), 8.08 (d, 1H, *J* = 8.0 Hz, H-7), 8.27 (d, 1H, *J* = 8.0 Hz, H-5), 8.77 (s, 1H, H-2); ^{13}C NMR (125 MHz, DMSO-*d*$_6$) δ 61.1 (C-5'), 70.1 (C-3'), 74.1 (C-2'), 86.0 (C-4'), 89.1 (C-1'), 118.7 (C-7), 118.8 (C-5), 122.3 (C-6), 135.5 (C-9), 136.9 (C-8), 138.8 (C-4), 145.8 (C-2); HRMS for $C_{12}H_{14}N_3O_6$ ($[M+H]^+$) calcd: 296.0877 found 296.0880.

General procedure for synthesis of 2',3',5'-tri-*O*-TBDMS nucleosides (3.8a-f)[134]

To a stirred solution of the nucleoside (1 mmol) and imidazole (8 mmol) in DMF (5 mL) was added TBDMSCl (4 mmol). The reaction mixture was stirred at 50 ^{o}C for 3 days. After completion of reaction, DMF was evaporated and the residue was partitioned between ethyl acetate (50 mL) and saturated $NaHCO_3$ (50 mL). The layers were separated and the aqueous layer was extracted with ethyl acetate (50 mLx3). The combined organic

layers were washed with brine, dried over sodium sulfate, filtered and evaporated to yield an oily residue which was purified by silica gel chromatography yield persilyated derivatives in 92-98% yield.

1-β-D-(4-nitro-benzimidazol-1-yl)-2',3',5'-tri-*O*-TBDMS-ribofuranoside (3.8a)

^{1}H NMR (500 MHz, $CDCl_3$) δ -0.68 (s, 3H, CH_3-Si), -0.16 (s, 3H, CH_3-Si), 0.12 (s, 3H, CH_3-Si), 0.13 (s, 3H, CH_3-Si), 0.17 (s, 3H, CH_3-Si), 0.19 (s, 3H, CH_3-Si), 0.71 (s, 9H, ^{t}Bu CH_3), 0.96 (s, 9H, ^{t}Bu CH_3), 0.98 (s, 9H, ^{t}Bu CH_3), 3.84-3.87 (dd, 1H, *J* = 2.0 Hz and 11.5 Hz, H-5'a), 3.95-3.99 (dd, 1H, *J* = 2.0 Hz and 11.5 Hz, H-5'b), 4.16-4.18 (m, 1H, H-4'), 4.23 (d, 1H, *J* = 4.5 Hz, H-3'), 4.40-4.44 (dd, 1H, *J* = 4.5 Hz and 7.5 Hz, H-2'), 5.93 (d, 1H, *J* = 8.0 Hz, H-1'), 7.35 (t, 1H, *J* = 8.0 Hz, H-6), 8.13 (d, 1H, *J* = 8.0 Hz, H-7), 8.18 (d, 1H, *J* = 8.0 Hz, H-5), 8.34 (s, 1H, H-2); ^{13}C NMR (125 MHz, $CDCl_3$) δ -6.7 (*C*H_3-Si), -6.3 (*C*H_3-Si), -6.2 (*C*H_3-Si), -5.5 (*C*H_3-Si), -5.4 (*C*H_3-Si), -5.3 (*C*H_3-Si), 16.9 (^{t}Bu *C*$(CH_3)_3$), 17.2 (^{t}Bu *C*$(CH_3)_3$), 17.7 (^{t}Bu *C*$(CH_3)_3$), 24.8 (^{t}Bu, *C*H_3), 25.0 (^{t}Bu, *C*H_3), 25.21 (^{t}Bu, *C*H_3), 62.6 (C-5'), 72.0 (C-3'), 74.9 (C-2'), 86.7 (C-4'), 88.3 (C-1'), 117.8 (C-7), 118.9 (C-5), 121.3 (C-6), 134.7 (C-9), 136.9 (C-8), 138.5 (C-4), 144.2 (C-2); HRMS for $C_{30}H_{56}N_3O_6Si_3$ ([M+H]$^+$) calcd: 638.3471 found 638.3482.

2',3',5'-tri-*O*-TBDMS-guanosine (3.8c)[135]

^{1}H NMR (300 MHz, DMSO-d_6) δ -0.28 (s, 3H, CH_3-Si), -0.08 (s, 3H, CH_3-Si), 0.09-0.12 (ms, 12H, 4xCH_3-Si), 0.73 (s, 9H, ^{t}Bu CH_3), 0.9 (2s, 18H, ^{t}Bu CH_3), 3.68-3.73 (dd, 1H, *J* = 3.9 Hz, and 11.4 Hz, H-5'a), 3.82-3.88 (dd, 1H, *J* = 5.4 Hz, 11.4 Hz, H-5'b), 3.93-3.95 (m, 1H, H-4'), 4.17 (d, 1H, *J* = 4.2 Hz, H-3'), 4.56-4.61 (dd, 1H, *J* = 4.5 Hz and 6.9 Hz, H-2'), 5.74 (d, 1H, *J* = 7.2 Hz, H-1'), 6.45 (bs, 2H, NH_2), 7.89 (s, 1H, H-8), 10.61 (bs, N*H*); ^{13}C NMR (75 MHz, DMSO-d_6) δ -5.39 (3x*C*H_3-Si), -4.67 (*C*H_3-Si), -4.64 (*C*H_3-Si), -4.55 (*C*H_3-Si), 17.7 (^{t}Bu *C*$(CH_3)_3$), 17.9 (^{t}Bu *C*$(CH_3)_3$), 18.2 (^{t}Bu *C*$(CH_3)_3$), 25.6 (^{t}Bu *C*H_3), 25.9 (^{t}Bu *C*H_3), 26.0 (^{t}Bu *C*H_3), 63.1 (C-5'), 72.9 (C-3'), 75.4 (C-2'), 85.4 (C-4'), 85.8 (C-1'), 116.7 (C-5), 135.0 (C-8), 151.8 (C-4), 153.9 (C-2), 156.8 (C-6); HRMS calcd for $C_{28}H_{56}N_5O_5Si_3$ ([M+H]$^+$) calcd: 626.3584 found: 626.3582.

2',3',5'-tri-*O*-TBDMS-inosine (3.8d)[136]

^{1}H NMR (300 MHz, DMSO-d_6) δ -0.53 (s, 3H, CH_3-Si), -0.13 (s, 3H, CH_3-Si), -0.02 (s, 3H, CH_3-Si), 0.01 (2s, 6H, 2xCH_3-Si), 0.74 (s, 9H, ^{t}Bu, CH_3), 0.94 (s, 9H, ^{t}Bu, CH_3), 3.71 (d, 1H, *J* = 12.6 Hz, H-5'), 3.94 (d, 1H, *J* = 12.9 Hz, H-5'), 4.15 (bs, 1H, H-4'), 4.37 (d, 1H, *J* = 4.2 Hz, H-3'), 4.82-4.92 (m, 1H, H-2'), 3.78 (d, 1H, *J* = 7.5 Hz, H-1'), 7.90 (s, 1H, H-8), 8.43 (s, 1H, H-2); ^{13}C NMR (75 MHz, DMSO-d_6) δ -5.7 (*C*H_3-Si), -4.52 (*C*H_3-Si), -4.47 (*C*H_3-Si), -4.4 (*C*H_3-Si), 17.9 (^{t}Bu *C*$(CH_3)_3$), 18.2 (^{t}Bu *C*$(CH_3)_3$), 25.8 (^{t}Bu *C*H_3), 25.9 (^{t}Bu *C*H_3), 62.9 (C-5'), 73.9 (C-3'), 74.8 (C-2'), 89.2 (C-4'), 91.0 (C-1'), 126.7 (C-5), 140.9 (C-8), 146.3 (C-2), 147.9 (C-4), 159.6 (C-6); HRMS for $C_{28}H_{55}N_4O_5Si_3$ ([M+H]$^+$) calcd: 611.3475 found 611.3472.

2',3',5'-tri-*O*-TBDMS-cytidine (3.8e)[134b]

^{1}H NMR (300 MHz, DMSO-d_6) δ -0.01 (s, 6H, 2xCH_3-Si), 0.06 (s, 3H, CH_3-Si), 0.07 (s, 3H, CH_3-Si), 0.09 (s, 3H, CH_3-Si), 0.10 (s, 3H, CH_3-Si), 0.83 (s, 9H, ^{t}Bu CH_3), 0.88 (s, 9H, ^{t}Bu CH_3), 0.91 (s, 9H, ^{t}Bu CH_3), 3.67-3.75 (m, 1H, H-5'a), 3.85-3.95 (m, 2H, H-5'b, H-4'), 4.04 (t, 1H, *J* = 4.2 Hz, H-3'), 4.10 (t, 1H, *J* = 4.5 Hz, H-2'), 5.71 (d, 1H, *J* = 7.5 Hz, H-5), 5.82 (d, 1H, *J* = 4.5 Hz, H-1'), 7.17 (bs, 2H, NH_2), 7.79 (d, 1H, *J* = 7.5 Hz, H-6); ^{13}C NMR (75 MHz, DMSO-d_6) δ -5.7 (*C*H_3-Si), -5.6 (*C*H_3-Si), -5.0 (2x*C*H_3-Si), -4.8 (*C*H_3-Si), -4.6 (*C*H_3-Si), 17.6 (^{t}Bu *C*$(CH_3)_3$), 17.7 (^{t}Bu *C*$(CH_3)_3$), 18.0 (^{t}Bu *C*$(CH_3)_3$), 25.6 (^{t}Bu *C*H_3), 25.7 (^{t}Bu *C*H_3), 25.8 (^{t}Bu *C*H_3), 61.9 (C-5'), 71.0 (C-3'), 75.2 (C-2'), 83.6 (C-4'), 87.8 (C-5), 93.8 (C-1'), 140.4 (C-6), 155.1 (C-2), 165.4 (C-4); HRMS for $C_{27}H_{56}N_3O_5Si_3$ ([M+H]$^+$) calcd: 586.3522 found 586.3522.

2',3',5'-tri-*O*-TBDMS-uridine (3.8f)

^{1}H NMR (300 MHz, DMSO-d_6) δ -0.02 (s, 3H, CH_3-Si), 0.03 (s, 3H, CH_3-Si), 0.09 (s, 3H, CH_3-Si), 0.09-0.11 (m, 9H, 3xCH_3-Si), 0.84 (s, 9H, ^{t}Bu CH_3), 0.89 (s, 9H, ^{t}Bu CH_3), 0.91 (s, 9H, ^{t}Bu CH_3), 3.68-3.75 (dd, 1H, *J* = 2.4 Hz and 11.4 Hz, H-5'a), 3.83-3.90 (dd, 1H, *J* = 3.9 Hz and 11.4 Hz, H-5'b), 3.93-3.97 (m, 1H, H-4'), 4.05-4.09 (m, 1H, H-3'), 4.20-4.25 (m, 1H, H-2'), 5.64 (d, 1H, *J* = 7.8 Hz, H-5), 5.82 (d, 1H, *J* = 5.7 Hz, H-1'), 7.77 (d, 1H, *J* = 8.1 Hz, H-6), 11.40 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6) δ -5.7 (*C*H_3-Si), -5.6 (*C*H_3-Si), -5.04 (*C*H_3-Si), -5.0 (*C*H_3-Si), -4.8 (*C*H_3-Si), -4.7 (*C*H_3-Si), 17.6 (^{t}Bu *C*$(CH_3)_3$), 17.7 (^{t}Bu *C*$(CH_3)_3$), 18.0 (^{t}Bu *C*$(CH_3)_3$), 25.5 (^{t}Bu *C*H_3), 25.7 (^{t}Bu *C*H_3), 25.8 (^{t}Bu *C*H_3), 62.3 (C-5'), 71.7 (C-3'), 74.4 (C-2'), 84.7 (C-4'), 86.9 (C-1'), 101.9 (C-5), 139.9 (C-6), 150.6 (C-2), 162.8 (C-4); HRMS for $C_{27}H_{53}N_2O_6Si_3$ ([M-H]$^-$) calcd: 585.3218 found 585.3218.

General procedure for 5'-desilylation (3.9a-f)[137]

To a stirred solution of the persilylated nucleoside (200 mg) in THF (4 mL) was added aqueous TFA (2 mL, TFA:water 1:1) at 0 ^{o}C. After stirring for 6 h at 0 ^{o}C, the reaction mixture was neutralized with saturated aqueous $NaHCO_3$ and diluted with ethyl acetate (80 mL). The layers were separated and the organic phase was washed with water (10 mL) and brine (10 mL), dried over anhydrous Na_2SO_4, filtered and evaporated at reduced pressure. The residue was subjected to silica gel chromatography to provide the 2',3'-disilylated products as a white solid in 90-99% yield.

1-(β-D-ribofuranosyl)-4-nitro-2',3'-di-*O*-TBDMS-benzimidazole (3.9a)

^{1}H NMR (300 MHz, $CDCl_3$) δ -0.54 (s, 3H, CH_3-Si), -0.09 (s, 3H, CH_3-Si), 0.15 (s, 6H, 2xCH_3-Si), 0.72 (s, 9H, ^{t}Bu CH_3), 1.0 (s, 9H, ^{t}Bu CH_3), 4.07-4.13 (m, 2H, H-5'a and H-5'b), 4.26 (bs, 1H, H-4'), 4.41 (d, 1H, *J* = 3.3 Hz, H-3'), 4.74-4.80 (dd, 1H, *J* = 4.8 Hz and 7.2 Hz, H-2'), 6.03 (d, 1H, *J* = 6.6 Hz, H-1'), 7.43 (t, 1H, *J* = 8.4 Hz, H-6), 7.92 (d, 1H, *J* = 8.4 Hz, H-7), 8.22 (d, 1H, *J* = 8.1 Hz, H-5), 9.19 (s, 1H, H-2); ^{13}C NMR (75

MHz, $CDCl_3$) δ -5.4 (*C*H_3-Si), -4.4 (2x*C*H_3-Si), -4.3 (*C*H_3-Si), 17.9 (^{t}Bu *C*$(CH_3)_3$), 18.3 (^{t}Bu *C*$(CH_3)_3$), 25.8 (^{t}Bu *C*H_3), 26.0 (^{t}Bu *C*H_3), 62.0 (C-5'), 74.2 (C-3'), 78.3 (C-2'), 87.9 (C-4'), 89.5 (C-1'), 117.3 (C-7), 120.0 (C-5), 122.6 (C-6), 136.4 (C-9), 136.6 (C-8), 139.1 (C-4), 145.5 (C-2); HRMS for $C_{24}H_{41}N_3O_6Si_2Na$ ($[M+Na]^+$) calcd: 546.2426 found 546.2420.

2',3'-di-*O*-TBDMS-adenosine (3.9b)[92c]

^{1}H NMR (300 MHz, DMSO-d_6) δ -0.38 (s, 3H, C*H*$_3$-Si), -0.10 (s, 3H, C*H*$_3$-Si), 0.10 (s, 3H, C*H*$_3$-Si), 0.12 (s, 3H, C*H*$_3$-Si), 0.72 (s, 9H, ^{t}Bu C*H*$_3$), 0.92 (s, 9H, ^{t}Bu C*H*$_3$), 3.55-3.63 (m, 1H, H-5'a), 3.70-3.80 (m, 1H, H-5'b), 3.95-4.02 (m, 1H, H-4'), 4.29-4.35 (m, 1H, H-3'), 4.75-4.83 (m, 1H, H-2'), 5.94 (d, 1H, *J* = 6.3 Hz, H-1'), 8.35 (s, 1H, H-2), 8.60 (s, 1H, H-8); ^{13}C NMR (75 MHz, DMSO-d_6) δ -5.7 (*C*H_3-Si), -4.9 (*C*H_3-Si), -4.8 (*C*H_3-Si), -4.7 (*C*H_3-Si), 17.5 (^{t}Bu *C*$(CH_3)_3$), 17.8 (^{t}Bu *C*$(CH_3)_3$), 25.5 (^{t}Bu *C*H_3), 25.7 (^{t}Bu *C*H_3), 61.0 (C-5'), 72.6 (C-3'), 74.8 (C-2'), 86.9 (C-4'), 87.5 (C-1'), 119.1 (C-5), 141.2 (C-8), 148.6 (C-4), 148.8 (C-2), 153.2 (C-6); HRMS for $C_{22}H_{42}N_5O_4Si_2$ ($[M+H]^+$) calcd: 496.2770 found 496.2771.

2',3'-di-*O*-TBDMS-guanosine (3.9c)[135]

^{1}H NMR (300 MHz, DMSO-d_6) δ -0.36 (s, 3H, C*H*$_3$-Si), -0.09 (s, 3H, C*H*$_3$-Si), 0.11 (s, 3H, C*H*$_3$-Si), 0.13 (s, 3H, C*H*$_3$-Si), 0.72 (s, 9H, ^{t}Bu C*H*$_3$), 0.92 (s, 9H, ^{t}Bu C*H*$_3$), 3.53-3.64 (m, 1H, H-5'a), 3.68-3.78 (m, 1H, H-5'b), 3.93-4.0 (m, 1H, H-4'), 4.27-4.32 (m, 1H, H-3'), 4.73 (dd, 1H, *J* = 4.2 Hz and 6.6 Hz, H-2'), 5.24 (t, 1H, *J* = 6.0 Hz, 5'-OH), 5.90 (d, 1H, *J* = 6.9 Hz, H-1'), 8.11 (H-8), 8.39 (NH); ^{13}C NMR (75 MHz, DMSO-d_6) δ -5.7 (*C*H_3-Si), -4.84 (*C*H_3-Si), -4.76 (*C*H_3-Si), -4.7 (*C*H_3-Si), 17.5 (^{t}Bu *C*$(CH_3)_3$), 17.8 (^{t}Bu *C*$(CH_3)_3$), 25.4 (^{t}Bu *C*H_3), 25.7 (^{t}Bu *C*H_3), 61.0 (C-5'), 72.7 (C-3'), 75.2 (C-2'), 86.7 (C-4'), 86.8 (C-1'), 124.5 (C-5), 138.9 (C-8), 146.0 (C-4), 148.2 (C-2), 156.5 (C-6); HRMS for $C_{22}H_{42}N_5O_5Si_2$ ($[M+H]^+$) calcd: 512.2718 found 512.2715.

2',3'-di-*O*-TBDMS-inosine (3.9d)

^{1}H NMR (300 MHz, $CDCl_3$) δ -0.53 (s, 3H, C*H*$_3$-Si), -0.13 (s, 3H, C*H*$_3$-Si), -.02 (s, 3H, C*H*$_3$-Si), 0.01 (2s, 6H, 2xC*H*$_3$-Si), 0.74 (s, 9H, ^{t}Bu, C*H*$_3$), 0.94 (s, 9H, ^{t}Bu, C*H*$_3$), 3.71 (d, 1H, *J* = 12.6 Hz, H-5'), 3.94 (d, 1H, *J* = 12.9 Hz, H-5'), 4.15 (bs, 1H, H-4'), 4.37 (d, 1H, *J* = 4.2 Hz, H-3'), 4.82-4.92 (m, 1H, H-2'), 5.78 (d, 1H, *J* = 7.5 Hz, H-1'), 7.90 (s, 1H, H-2), 8.43 (s, 1H, H-8); ^{13}C NMR (75 MHz, $CDCl_3$) δ -5.7 (*C*H_3-Si), -4.52 (*C*H_3-Si), -4.47 (*C*H_3-Si), -4.4 (*C*H_3-Si), 17.9 (^{t}Bu *C*$(CH_3)_3$), 18.2 (^{t}Bu *C*$(CH_3)_3$), 25.8 (^{t}Bu *C*H_3), 25.9 (^{t}Bu *C*H_3), 62.9 (C-5'), 73.9 (C-3'), 74.8 (C-2'), 89.2 (C-4'), 91.0 (C-1'), 126.7 (C-5), 140.9 (C-8), 146.3 (C-2), 147.9 (C-4), 159.6 (C-6); HRMS for $C_{22}H_{41}N_4O_5Si_2$ ($[M+H]^+$) calcd: 497.2610 found 497.2608.

2',3'-di-*O*-TBDMS-cytidine (3.9e)

^{1}H NMR (600 MHz, DMSO-d_6) δ -0.01 (s, 3H, CH_3-Si), 0.01 (s, 3H, CH_3-Si), 0.06 (s, 3H, CH_3-Si), 0.08 (s, 3H, CH_3-Si), 0.82 (s, 9H, ^{t}Bu CH_3), 0.88 (s, 9H, ^{t}Bu CH_3), 3.51-3.56 (m, 1H, H-5'a), 3.65-3.70 (m, 1H, H-5'b), 3.86 (q, 1H, *J* = 3.6 Hz, H-4'), 4.10 (t, 1H, *J* = 4.2 Hz, H-3'), 4.19 (t, 1H, *J* = 4.8 Hz, H-2'), 5.19-5.21 (m, 1H, 5'-OH) 5.75 (d, 1H, *J* = 7.2 Hz, H-5) 5.77 (d, 1H, *J* = 4.8 Hz, H-1') 7.20-7.35 (m, 2H, NH_2), 7.91 (d, 1H, *J* = 7.2 Hz, H-6); ^{13}C NMR (150 MHz, DMSO-d_6) δ -4.9 (2x*C*H_3-Si), -4.8 (*C*H_3-Si), -4.6 (*C*H_3-Si), 17.7 (^{t}Bu *C*$(CH_3)_3$), 17.8 (^{t}Bu *C*$(CH_3)_3$), 25.7 (^{t}Bu *C*H_3), 25.8 (^{t}Bu *C*H_3), 60.1 (C-5'), 71.4 (C-3'), 74.9 (C-2'), 84.5 (C-4'), 88.3 (C-5), 94.0 (C-1'), 141.5 (C-6), 155.0 (C-2), 165.2 (C-4); HRMS for $C_{22}H_{42}N_3O_5Si_2$ ([M-H]$^-$) calcd: 472.2657 found 472.2652.

2',3'-di-*O*-TBDMS-uridine (3.9f)[92c]

^{1}H NMR (300 MHz, DMSO-d_6) δ -0.03 (s, 3H, CH_3-Si), 0.02 (s, 3H, CH_3-Si), 0.08 (s, 3H, CH_3-Si), 0.09 (s, 3H, CH_3-Si), 0.83 (s, 9H, ^{t}Bu CH_3), 0.88 (s, 9H, ^{t}Bu CH_3), 3.52-3.70 (m, 2H, H-5'a and H-5'b), 3.85-3.90 (m, 1H, 5'-OH), 4.10-4.17 (m, 1H, H-3'), 4.25 (t, 1H, *J* = 5.7 Hz, H-2'), 5.24 (d, 1H, *J* = 4.8 Hz, H-4') 5.68 (d, 1H, *J* = 8.4 Hz, H-5) 5.81 (d, 1H, *J* = 6.0 Hz, H-1') 7.93 (d, 1H, *J* = 8.1 Hz, H-6), 11.34 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6) δ -5.0 (*C*H_3-Si), -4.9 (*C*H_3-Si), -4.8 (*C*H_3-Si), -4.6 (*C*H_3-Si), 17.6 (^{t}Bu *C*$(CH_3)_3$), 17.7 (^{t}Bu *C*$(CH_3)_3$), 25.59 (^{t}Bu *C*H_3), 25.64 (^{t}Bu *C*H_3), 60.4 (C-5'), 71.9 (C-3'), 74.6 (C-2'), 85.5 (C-4'), 86.9 (C-1'), 102.0 (C-5), 140.3 (C-6), 150.8 (C-2), 163.0 (C-4); HRMS for $C_{21}H_{39}N_2O_6Si_2$ ([M-H]$^-$) calcd: 471.2352 found 471.2343.

General procedure for synthesis of the respective 5'-*O*-sulfamoyl nucleosides (3.10a-f)

Formic acid (2.5 mmol) was added to ice cooled chlorosulfonyl isocyanate (2.5 mmol) and allowed to stir at 0 ^{o}C for 5 min. The resulting solid was dissolved in dry acetonitrile (2 mL) and cooled to 0 ^{o}C. The obtained sulfamoyl chloride was then added to an ice cooled solution of the respective nucleoside (1 mmol) in dimethyl acetamide (5 mL) and allowed to stir at room temperature for 1 h. After 1 h, TEA (1.5 mL, excess) was added and stirring was continued for an additional 10 min. Subsequently, methanol (2 mL) was added and the mixture was stirred for an additional 15 min. Finally, the solvent was evaporated to dryness and the residue was dissolved in ethyl acetate and washed with saturated $NaHCO_3$. The organic layer was further washed with water and brine. The organic layer was collected, dried over Na_2SO_4, filtered and evaporated. The residue was subjected to silica gel chromatography to afford sulfamoylated nucleoside in 63-80% yield.

1-(2',3'-di-*O*-TBDMS)-5'-*O*-sulfamoyl-β-D-ribofuranosyl)-4-nitro-benzimidazole (3.10a)

^{1}H NMR (500 MHz, $CDCl_3$) δ -0.50 (s, 3H, CH_3-Si), -0.10 (s, 3H, CH_3-Si), 0.13 (s, 6H, 2xCH_3-Si), 0.71 (s, 9H, ^{t}Bu CH_3), 0.95 (s, 9H, ^{t}Bu CH_3), 4.31-4.36 (m, 2H, H-5'a and H-5'b), 4.41-4.45 (m, 2H, H-4', H-3'), 4.55-4.59 (dd, 1H, *J* = 2.5 Hz, 11.0 Hz, H-2'), 5.97 (d, 1H, *J* = 6.5 Hz, H-1'), 6.68 (bs, 2H, NH_2), 7.40 (t, 1H, *J* = 8.0 Hz, H-6), 7.85 (d, 1H, *J* = 8.0 Hz, H-7), 8.15 (d, 1H, *J* = 8.0 Hz, H-5), 8.59 (s, 1H, H-2); ^{13}C NMR (125 MHz, $CDCl_3$) δ -6.4 (*C*H_3-Si), -5.6 (*C*H_3-Si), -5.5 (*C*H_3-Si), -5.3 (*C*H_3-Si), 16.9 (^{t}Bu *C*$(CH_3)_3$), 17.1 (^{t}Bu *C*$(CH_3)_3$), 24.8 (^{t}Bu *C*H_3), 25.0 (^{t}Bu *C*H_3), 67.9 (C-5'), 71.9 (C-2'), 76.0 (C-3'), 82.9 (C-4'), 88.0 (C-1'), 116.5 (C-7), 119.1 (C-5), 121.8 (C-6),134.9 (C-9), 135.8 (C-8), 138.1 (C-4), 143.7 (C-2); HRMS for $C_{24}H_{43}N_4O_8SSi_2$ ([M+H]$^+$) calcd: 603.2334 found 603.2325.

2',3'-di-*O*-TBDMS-5'-*O*-sulfamoyl-adenosine (3.10b)

^{1}H NMR (300 MHz, DMSO-d_6) δ -0.37 (s, 3H, CH_3-Si), -0.08 (s, 3H, CH_3-Si), 0.13 (s, 3H, CH_3-Si), 0.15 (s, 3H, CH_3-Si), 0.71 (s, 9H, ^{t}Bu CH_3), 0.93 (s, 9H, ^{t}Bu CH_3), 4.15-4.21 (m, 1H, H-3'), 4.27-4.34 (m, 1H, H-2'), 4.36-4.44 (m, 2H, H-5'a and H-5'b), 4.94-4.94 (dd, 1H, *J* = 4.5 Hz, 6.6 Hz, H-4'), 5.96 (d, 1H, *J* = 6.9 Hz, H-1'), 7.31 (s, 2H, NH_2), 7.64 (s, 2H, NH_2), 8.16 (s, 1H, H-8), 8.36 (s, 1H, H-2); ^{13}C NMR (75 MHz, DMSO-d_6) δ -5.6 (*C*H_3-Si), -4.9 (*C*H_3-Si), -4.8 (*C*H_3-Si), -4.7 (*C*H_3-Si), 17.5 (^{t}Bu *C*$(CH_3)_3$), 17.7 (^{t}Bu *C*$(CH_3)_3$), 25.4 (^{t}Bu *C*H_3), 25.7 (^{t}Bu *C*H_3), 68.0 (C-5'), 72.4 (C-2'), 73.7 (C-3'), 82.8 (C-4'), 87.0 (C-1'), 119.3 (C-5), 139.8 (C-8), 149.4 (C-4), 152.7 (C-2), 156.1 (C-6); HRMS for $C_{22}H_{43}N_6O_6SSi_2$ ([M+H]$^+$) calcd: 575.2498 found 575.2530.

2',3'-di-*O*-TBDMS-5'-*O*-sulfamoyl-guanosine (3.10c)

^{1}H NMR (300 MHz, DMSO-d_6) δ -0.28 (s, 3H, CH_3-Si), -0.06 (s, 3H, CH_3-Si), 0.12 (s, 3H, CH_3-Si), 0.14 (s, 3H, CH_3-Si), 0.73 (s, 9H, ^{t}Bu CH_3), 0.92 (s, 9H, ^{t}Bu CH_3), 4.12-4.17 (m, 1H, H-3'), 4.21-4.35 (m, 3H, H-2', H-5'a and H-5'b), 4.75-4.81 (dd, 1H, *J* = 4.5 Hz, 7.2 Hz, H-4'), 5.76 (d, 1H, *J* = 7.5 Hz, H-1'), 6.47 (bs, 2H, $SONH_2$), 7.66 (s, 2H, 2-NH_2), 7.91 (s, 1H, H-8), 10.69 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6) δ -5.6 (*C*H_3-Si), -4.9 (*C*H_3-Si), -4.7 (2x*C*H_3-Si), 17.5 (^{t}Bu *C*$(CH_3)_3$), 17.7 (^{t}Bu *C*$(CH_3)_3$), 25.5 (^{t}Bu *C*H_3), 25.7 (^{t}Bu *C*H_3), 68.1 (C-5'), 72.7 (C-2'), 74.0 (C-3'), 83.0 (C-4'), 85.8 (C-1'), 116.8 (C-5), 135.6 (C-8), 151.5 (C-4), 153.7 (C-2), 156.7 (C-6); HRMS for $C_{22}H_{43}N_6O_7SSi_2$ ([M+H]$^+$) calcd: 591.2447 found 591.2455.

2',3'-di-*O*-TBDMS-5'-*O*-sulfamoyl-inosine (3.10d)

^{1}H NMR (300 MHz, $CDCl_3$) δ -0.33 (s, 3H, CH_3-Si), -0.08 (s, 3H, CH_3-Si), 0.11 (s, 3H, CH_3-Si), 0.15 (s, 3H, CH_3-Si),0.76 (s, 9H, ^{t}Bu CH_3), 0.94 (s, 9H, ^{t}Bu CH_3), 4.30-4.42 (m, 2H, H-5'a and H-5'b), 4.48-4.52 (m, 1H, H-4'), 4.65-4.75 (m, 1H, H-3'), 4.75-4.80 (m, 1H, H-2'), 5.82 (d, 1H, *J* = 5.4 Hz, H-1'), 7.01 (bs, 2H, NH_2), 8.01 (s, 1H, H-8), 8.42 (s,

1H, H-2); ^{13}C NMR (75 MHz, $CDCl_3$) δ -5.1 (*C*H_3-Si), -4.7 (*C*H_3-Si), -4.6 (*C*H_3-Si), -4.3 (*C*H_3-Si), 17.9 (^{t}Bu *C*$(CH_3)_3$), 18.2 (^{t}Bu *C*$(CH_3)_3$), 25.8 (^{t}Bu *C*H_3), 26.0 (^{t}Bu *C*H_3), 69.0 (C-5'), 72.3 (C-2'), 74.8 (C-3'), 83.3 (C-4'), 90.0 (C-1'), 124.8 (C-5), 140.8 (C-8), 146.1 (C-2), 148.2 (C-4), 158.3 (C-6); HRMS for $C_{22}H_{41}N_5O_7SSi_2$ ($[M+H]^+$) calcd: 576.2338 found 576.2347.

2',3'-di-*O*-TBDMS-5'-*O*-sulfamoyl-cytidine (3.10e)

^{1}H NMR (300 MHz, DMSO-d_6) δ 0.00 (s, 3H, C*H*$_3$-Si), 0.02 (s, 3H, C*H*$_3$-Si), 0.08 (s, 3H, C*H*$_3$-Si), 0.10 (s, 3H, C*H*$_3$-Si),0.83 (s, 9H, ^{t}Bu C*H*$_3$), 0.88 (s, 9H, ^{t}Bu C*H*$_3$), 4.04-4.18 (m, 3H, H-5'a, H-5'b and H-4'), 4.22-4.32 (m, 2H, H-2' and H-3'), 5.73-5.80 (m, 2H, H-5 and H-1'), 7.21-7.38 (m, 2H, SON*H*$_2$), 7.63 (d, 1H, *J* = 7.5 Hz, H-6), 7.68 (s, 2H, N*H*$_2$); ^{13}C NMR (75 MHz, DMSO-d_6) δ -5.0 (*C*H_3-Si), -4.9 (*C*H_3-Si), -4.8 (*C*H_3-Si), -4.6 (*C*H_3-Si), 17.6 (^{t}Bu *C*$(CH_3)_3$), 17.7 (^{t}Bu *C*$(CH_3)_3$), 25.67 (^{t}Bu *C*H_3), 25.69 (^{t}Bu *C*H_3), 67.5 (C-5'), 71.2 (C-3'), 74.0 (C-2'), 81.0 (C-4'), 88.9 (C-5), 94.3 (C-1'), 141.1 (C-6), 154.8 (C-2), 165.2 (C-4); HRMS for $C_{21}H_{43}N_4O_7SSi_2$ ($[M+H]^+$) calcd: 551.2385 found 551.2380.

2',3'-di-*O*-TBDMS-5'-*O*-sulfamoyl-uridine (3.10f)

^{1}H NMR (300 MHz, $CDCl_3$) δ 0.07 (s, 3H, C*H*$_3$-Si), 0.08 (s, 3H, C*H*$_3$-Si), 0.10 (s, 3H, C*H*$_3$-Si), 0.11 (s, 3H, C*H*$_3$-Si),0.88 (s, 9H, ^{t}Bu C*H*$_3$), 0.91 (s, 9H, ^{t}Bu C*H*$_3$), 4.12 (t, 1H, *J* = 4.2 Hz, H-5'a) 4.24 (q, 1H, *J* = 3.9 Hz, H-4'), 4.28 (dd, 1H, *J* = 3.6 Hz and 11.1 Hz, H-5'b), 4.48-4.54 (m, 2H, H-2' and H-3'), 5.53 (d, 1H, *J* = 4.5 Hz, H-1'), 5.62 (bs, 2H, SON*H*$_2$), 5.77 (d, 1H, *J* = 8.1 Hz, H-5), 7.50 (d, 1H, *J* = 8.1 Hz, H-6), 9.70 (bs, 1H, N*H*); ^{13}C NMR (75 MHz, $CDCl_3$) δ -5.8 (*C*H_3-Si), -5.59 (*C*H_3-Si), -5.56 (*C*H_3-Si), -5.2 (*C*H_3-Si), 17.10 (^{t}Bu *C*$(CH_3)_3$), 17.15 (^{t}Bu *C*$(CH_3)_3$), 24.90 (^{t}Bu *C*H_3), 24.93 (^{t}Bu *C*H_3), 67.4 (C-5'), 70.5 (C-3'), 72.6 (C-2'), 81.1 (C-4'), 92.1 (C-1'), 101.6 (C-5), 141.1 (C-6), 149.7 (C-2), 162.7 (C-4); HRMS for $C_{21}H_{41}N_3O_8SSi_2Na$ ($[M+Na]^+$) calcd: 574.2045 found 574.2043.

General procedure for synthesis of 5'-*O*-(*N*-L-isoleucyl)-sulfamoyl nucleosides (3.11a-f)

To a solution of the respective 2',3'-di-*O*-(tert-butyldimethylsilyl)-5'-*O*-sulfamoyl nucleoside (1.0 mmol) and N^{α}-Boc-L-isoleucine *N*-hydroxysuccinimide ester (1.1 equiv) in DMF (3 mL) was added DBU (1.1 equiv) and the reaction mixture was stirred at rt for 6-8h. Next, the volatiles were removed *in vacuo* and the residue was purified by silica gel chromatography (Et_3N 1%, MeOH 2.5-10% in CH_2Cl_2). The coupled product was next treated with TFA/H_2O (5/2 v/v) for 2 h at rt, after which the volatiles were evaporated and coevaporated twice with EtOH and once with EtOH and Et_3N (2 mL), to neutralize any remaining acid. The compound was carefully dried and dissolved in THF (2 mL) and Et_3N.3HF (0.5 mL). After 3 h, another 0.4 mL of Et_3N.3HF was added and the reaction mixture was stirred further for 22 h. The reaction mixture was evaporated and the residue was purified by flash chromatography (5 to 50% CH_2Cl_2:MeOH) and finally by HPLC

using PLRP-S 100 Å column and acetonitrile:water as mobile phase to yield isoleucyl-sulfamoyl nucleoside as white solid in 28-65% yield.

1-[5'-*O*-(*N*-L-isoleucyl-sulfamoyl-β-D-ribofuranosyl)]-4-nitro-benzimidazole (3.11a)

^{1}H NMR (500 MHz, D_2O) δ 0.74 (t, 3H, *J* = 10.0 Hz, Ile-δ-C*H*$_3$), 0.88 (d, 3H, *J* = 10.0 Hz, Ile-γ-C*H*$_3$), 1.05-1.15 (m, 1H, Ile-γ-C*H*$_2$ Ha), 1.32-1.41 (m, 1H, Ile-γ-C*H*$_2$ Hb), 1.79-1.85 (m, 1H, Ile-β-C*H*), 3.46 (d, 1H, *J* = 5.0 Hz, Ile-α-C*H*), 4.38-4.45 (m, 2H, H-5'a and H-5'b), 4.45-4.50 (m, 2H, H-3' and H-4'), 4.61 (t, 1H, *J* = 5.0 Hz, H-2'), 6.09 (d, 1H, *J* = 5.0 Hz, H-1'), 7.44 (t, 1H, *J* = 10.0 Hz, H-6), 8.03 (d, 1H, *J* = 10.0 Hz, H-7), 8.12 (d, 1H, *J* = 10.0 Hz, H-5) 8.60 (s, 1H, H-2); ^{13}C NMR (75 MHz, D_2O) δ 10.4 (Ile-δ-*C*H$_3$), 14.4 (Ile-γ-*C*H$_3$), 23.6 (Ile-δ-*C*H$_2$), 37.0 (Ile-β-*C*H), 60.3 (Ile-α-*C*H), 67.7 (C-5'), 69.8 (C-3'), 73.5 (C-2'), 82.5 (C-4'), 88.8 (C-1'), 118.7 (C-7), 120.2 (C-5), 123.0 (C-6), 134.5 (C-9), 135.6 (C-8), 137.3 (C-4), 145.0 (C-2), 178.4 (C=O, Ile); HRMS for $C_{18}H_{24}N_5O_9S$ ([M-H]$^-$) calcd: 486.1300 found 486.1301.

5'-*O*-(*N*-L-isoleucyl)-sulfamoyl-adenosine (3.11b)

^{1}H NMR (300 MHz, D_2O) δ 0.87 (t, 3H, *J* = 7.5 Hz, Ile-δ-C*H*$_3$), 0.98 (d, 3H, *J* = 6.9 Hz, Ile-γ-C*H*$_3$), 1.02-1.28 (m, 1H, Ile-γ-C*H*$_2$ Ha), 1.40-1.53 (m, 1H, Ile-γ-C*H*$_2$ Hb), 1.95-2.07 (m, 1H, Ile-β-C*H*), 3.76 (d, 1H, *J* = 4.2 Hz, Ile-α-C*H*), 4.43-4.51 (m, 3H, H-5'a, H-5'b and H-4'), 4.56 (t, 1H, *J* = 5.1 Hz, H-3'), 4.75-4.78 (m, 1H, merged with D2O peak, H-2'), 6.13 (d, 1H, *J* = 5.1 Hz, H-1'), 8.24 (s, 1H, H-8), 8.42 (s, 1H, H-2); ^{13}C NMR (75 MHz, D_2O) δ 11.5 (Ile-δ-*C*H$_3$), 15.1 (Ile-γ-*C*H$_3$), 24.7 (Ile-δ-*C*H$_2$), 37.0 (Ile-β-*C*H), 60.7 (Ile-α-*C*H), 68.9 (C-5'), 70.8 (C-3'), 74.7 (C-2'), 82.9 (C-4'), 87.9 (C-1'), 119.1 (C-5), 140.4 (C-8), 149.5 (C-4), 153.1 (C-2), 155.8 (C-6), 175.6 (C=O, Ile); HRMS for $C_{16}H_{24}N_7O_7S$ ([M-H]$^-$) calcd: 458.1463 found 458.1461.

5'-*O*-(*N*-L-isoleucyl)-sulfamoyl-guanosine (3.11c)

^{1}H NMR (600 MHz, D_2O) δ 0.83 (t, 3H, *J* = 7.2 Hz, Ile-δ-C*H*$_3$), 0.94 (d, 3H, *J* = 7.2 Hz, Ile-γ-C*H*$_3$), 1.01-1.19 (m, 1H, Ile-γ-C*H*$_2$ Ha), 1.34-1.43 (m, 1H, Ile-γ-C*H*$_2$ H$_b$), 1.92-1.99 (m, 1H, Ile-β-C*H*), 3.89 (d, 1H, *J* = 4.2 Hz, Ile-α-C*H*), 4.35-4.40 (m, 3H, H-5'a, H-5'b and H-4'), 4.50 (t, 1H, *J* = 4.2 Hz, H-3'), 4.70-4.75 (m, 1H, H-2'), 5.92 (d, 1H, *J* = 6.0 Hz, H-1'), 8.03 (s, 1H, H-8); ^{13}C NMR (125 MHz, D_2O) δ 10.5 (Ile-δ-*C*H$_3$), 14.1 (Ile-γ-*C*H$_3$), 23.7 (Ile-δ-*C*H$_2$), 36.0 (Ile-β-*C*H), 59.7 (Ile-α-*C*H), 68.0 (C-5'), 69.8 (C-3'), 73.2 (C-2'), 81.9 (C-4'), 86.6 (C-1'), 115.9 (C-5), 137.1 (C-8), 151.4 (C-4), 153.6 (C-2), 158.7 (C-6), 174.7 (C=O, Ile); HRMS for $C_{16}H_{24}N_7O_8S$ ([M-H]$^-$) calcd: 474.1412 found 474.1407.

5'-*O*-(*N*-L-isoleucyl)-sulfamoyl-inosine (3.11d)

^{1}H NMR (300 MHz, D_2O) δ 0.74 (t, 3H, *J* = 7.5 Hz, Ile-δ-C*H*$_3$), 0.86 (d, 3H, *J* = 7.2 Hz, Ile-γ-C*H*$_3$), 1.01-1.17 (m, 1H, Ile-γ-C*H*$_2$ Ha), 1.25-1.40 (m, 1H, Ile-γ-C*H*$_2$ H$_b$), 1.82-1.95 (m, 1H, Ile-β-C*H*), 3.62 (d, 1H, *J* = 4.2 Hz, Ile-α-C*H*), 4.29-4.38 (m, 3H, H-5'a, H-5'b and H-4'), 4.43 (t, 1H, *J* = 4.2 Hz, H-3'), 4.62-4.70 (m, 1H, merged with solvent peak, H-2'), 6.02 (d, 1H, *J* = 5.1 Hz, H-1'), 8.11 (s, 1H, H-8), 8.27 (s, 1H, H-2); ^{13}C NMR (75 MHz, D_2O) δ 10.6 (Ile-δ-*C*H$_3$), 14.1 (Ile-γ-*C*H$_3$), 23.7 (Ile-δ-*C*H$_2$), 36.1 (Ile-β-*C*H), 59.8 (Ile-α-*C*H), 67.8 (C-5'), 69.9 (C-3'), 73.9 (C-2'), 82.1 (C-4'), 87.4 (C-1'), 123.4 (C-5), 139.2 (C-8), 146.1 (C-2), 148.4 (C-4), 158.5 (C-6), 175.0 (C=O, Ile); HRMS for $C_{16}H_{23}N_6O_8S$ ([M-H]$^-$) calcd: 459.1303 found 459.1304.

5'-*O*-(*N*-L-isoleucyl)-sulfamoyl-cytidine (3.11e)

^{1}H NMR (300 MHz, D_2O) δ 0.91 (t, 3H, *J* = 7.5 Hz, Ile-δ-C*H*$_3$), 1.02 (d, 3H, *J* = 7.2 Hz, Ile-γ-C*H*$_3$), 1.17-1.33 (m, 1H, Ile-γ-C*H*$_2$ Ha), 1.41-1.56 (m, 1H, Ile-γ-C*H*$_2$ H$_b$), 1.99-2.10 (m, 1H, Ile-β-C*H*), 3.76 (d, 1H, *J* = 4.2 Hz, Ile-α-C*H*), 4.23-4.37 (m, 4H, H-5'a, H-5'b H-4'and H-3'), 4.44-4.51 (m, 1H, H-2'), 5.95 (d, 1H, *J* = 3.3 Hz, H-1'), 6.10 (d, 1H, *J* = 7.5 Hz, H-5) 7.83 (d, 1H, *J* = 7.8 Hz, H-6); ^{13}C NMR (75 MHz, D_2O) δ 10.6 (Ile-δ-*C*H$_3$), 14.2 (Ile-γ-*C*H$_3$), 23.8 (Ile-δ-*C*H$_2$), 36.1 (Ile-β-*C*H), 59.8 (Ile-α-*C*H), 67.6 (C-5'), 68.9 (C-3'), 73.7 (C-2'), 80.9 (C-4'), 89.5 (C-5), 96.1 (C-1'), 141.0 (C-6), 157.3 (C-2), 165.8 (C-4), 174.8 (C=O, Ile); HRMS for $C_{15}H_{26}N_5O_8S$ ([M+H]$^+$) calcd: 436.1496 found 436.1495.

5'-*O*-(*N*-L-isoleucyl)-sulfamoyl-uridine (3.11f)

^{1}H NMR (300 MHz, D_2O) δ 0.93 (t, 3H, *J* = 7.5 Hz, Ile-δ-C*H*$_3$), 1.02 (d, 3H, *J* = 6.9 Hz, Ile-γ-C*H*$_3$), 1.18-1.34 (m, 1H, Ile-γ-C*H*$_2$ Ha), 1.42-1.56 (m, 1H, Ile-γ-C*H*$_2$ H$_b$), 1.98-2.11 (m, 1H, Ile-β-C*H*), 3.75 (d, 1H, *J* = 3.9 Hz, Ile-α-C*H*), 4.38-4.48 (m, 5H, H-5'a, H-5'b, H-4' H-3', H-2'), 5.92 (m, 2H, H-1' and H-5), 7.84 (d, 1H, *J* = 8.1 Hz, H-6); ^{13}C NMR (75 MHz, D_2O) δ 10.7 (Ile-δ-*C*H$_3$), 14.2 (Ile-γ-*C*H$_3$), 23.8 (Ile-δ-*C*H$_2$), 36.1 (Ile-β-*C*H), 59.7 (Ile-α-*C*H), 67.7 (C-5'), 69.2 (C-3'), 73.3 (C-2'), 81.4 (C-4'), 88.5 (C-1'), 102.2 (C-5), 141.2 (C-6), 151.3 (C-2), 165.8 (C-4), 174.8 (C=O, Ile); HRMS for $C_{15}H_{23}N_4O_9S$ ([M-H]$^-$) calcd: 435.1191 found 435.1192.

5'-*O*-(*N*-L-isoleucyl)-sulfamoyl-1,3-dideaza-adenosine (3.11g)

To a solution of **3.11a** (50 mg, 0.1 mmol) in dry methanol (5 mL) was added Pd/C (10% w/w, 20 mg) and the mixture was stirred under H_2 atmosphere at room temperature for 7 h. Next, the catalyst was filtered off and the solvent was evaporated to dryness. The product was purified by silica gel column chromatography and finally by HPLC to yield 19 mg (0.04 mmol, 40%) of title compound as faint brown solid.

^{1}H NMR (500 MHz, D_2O) δ 0.80 (t, 3H, *J* = 7.5 Hz, Ile-δ-C*H*$_3$), 0.95 (d, 3H, *J* = 7.0 Hz, Ile-γ-C*H*$_3$), 1.12-1.24 (m, 1H, Ile-γ-C*H*$_2$ Ha), 1.37-1.46 (m, 1H, Ile-γ-C*H*$_2$ Hb), 1.95-2.02 (m, 1H, Ile-β-C*H*), 3.71 (d, 1H, *J* = 4.0 Hz, Ile-α-C*H*), 4.41-4.46 (m, 3H, H-5'a, H-5'b, H-4'), 4.48-4.51 (m,1H, H-3'), 4.67 (t, 1H, *J* = 6.0 Hz, H-2'), 6.04 (d, 1H, *J* = 6.5 Hz, H-1'), 6.78 (d, 1H, *J* = 7.5 Hz, H-1), 7.17 (d, 1H, *J* = 7.5 Hz, H-3), 7.23 (t, 1H, *J* = 8.0 Hz, H-2), 8.34 (s, 1H, H-8); ^{13}C NMR (125 MHz, D_2O) δ 10.5 (Ile-δ-*C*H$_3$), 14.1 (Ile-γ-*C*H$_3$), 23.7 (Ile-δ-*C*H$_2$), 36.0 (Ile-β-*C*H), 59.8 (Ile-α-*C*H), 68.1 (C-5'), 69.7 (C-3'), 72.8 (C-2'), 82.0 (C-4'), 88.2 (C-1'), 101.7 (C-3), 108.3 (C-1), 124.7 (C-2), 132.1 (C-5), 133.0 (C-6), 137.5 (C-4), 140.5 (C-8), 174.8 (C=O Ile); HRMS for $C_{18}H_{26}N_5O_7S$ ($[M-H]^-$) calcd: 456.1558 found 456.1560.

General procedure for synthesis of nucleoside sulfamate-hexapeptidyl conjugates (3.12a-g)[81]

The peptide formyl-methionyl-arginyl-(2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl)-threonyl(tBu)-glycyl-asparaginyl-(trityl)-alanyl-OH was synthesized on a 2-chlorotrityl chloride resin using standard Fmoc-based solid phase peptide chemistry. The protected hexapeptide was cleaved from the resin using a mixture of HOAc/trifluoroethanol/DCM (1/1/8, v/v) in 30 min. Following RP-HPLC purification, the peptide (20 mg, 16.13 µmol, 1.0 eq.) and HOBt (9 mg, 64.52 µmol, 4.0 eq.) were dissolved in DMF (500 µL) and DIC (10 µL, 64.52 µmol, 4.0 eq.) was added. This mixture was stirred for 1 h at rt under argon atmosphere. Following addition of DIPEA (7.5 µL, 40.33 µmol, 2.5 eq.), the mixture was added to the nucleoside sulfamate analogue **3.11a-g** (32.26 µmol, 2.0 eq.) and stirred for 16 h at rt under argon. Next, the volatiles were evaporated and the residue was taken up in a mixture of CH_3CN/water. This was purified on a PoraPak Rxn RP 6 cc Vac Cartridge 80µm particle (Waters®) column with a CH_3CN gradient of 25 to 100% in water). The fractions containing the product were evaporated and the protected conjugate was subsequently deprotected using a mixture of 90% TFA, 7.5% H_2O and 2.5% thioanisole. The reaction mixture was poured in chilled diethyl ether and centrifuged. Supernatant was decanted and the residue was re-suspended in chilled diethyl ether and centrifuged. A small amount of TEA (500 µL) was added for final washing. The product was dissolved in CH_3CN/water and purified by RP-HPLC (solvent A: 25 mM TEAB in H_2O; solvent B: 25 mM TEAB in CH_3CN; see supporting file for HPLC analysis of all final compounds). Overall yield for coupling, deprotection and tedious HPLC purification is generally low and affords about 6-12% yield.

fMRTGNAI-S[(4-nitrobenzimidazole)-β-ribofuranoside] (3.12a)

^{1}H NMR (600 MHz, D_2O) δ 0.73 (t, 3H, *J* = 7.2 Hz, Ile-δ-C*H*$_3$), 0.81 (d, 3H, *J* = 6.6 Hz, Ile-γ-C*H*$_3$), 1.00-1.10 (m, 1H, Ile-γ-C*H*$_2$, Ha), 1.14-1.21 (m, Thr-γ-C*H*$_3$, TEA-C*H*$_3$), 1.27-1.39 (d+m, 4H, *J* = 7.2 Hz, Ala-β-C*H*$_3$ and Ile-γ-C*H*$_2$, Hb), 1.55 (m, 2H, Arg-γ-C*H*$_2$), 1.67-1.88 (m, 3H, Ile β-C*H* and 2H, Arg-β-C*H*$_2$), 1.95-2.07 (m+s, 5H, Met-β-C*H*$_2$ and

Met-SCH_3), 2.53 (m, 2H, Met-γ-CH_2), 2.37 (dd, 1H, *J* = 8.4 Hz and 15.6 Hz, Asn-β-CH_2, Ha), 2.78 (dd, 1H, *J* = 4.8 Hz, and 16.2 Hz, Asn-β-CH_2, Hb), 3.00 (m, TEA-CH_2), 3.10 (m, 2H, Arg-δ-CH_2), 3.87 (d, 1H, *J* = 16.9 Hz, Gly-α-CH_2, Ha), 3.94-4.00 (m, 2H, Ile-α-C*H* and Gly-α-CH_2, Hb), 4.19-4.28 (m, 2H, Thr-β-C*H*, Ala-α-C*H*), 4.31-4.41 (m, 4H, Thr-α-C*H*, Arg-α-C*H* and H-5'a and H-5'b), 4.42-4.46 (m, 2H, H-3'and H-4'), 4.49 (dd, 1H, *J* = 5.9 Hz and 8.2 Hz, Met-α-C*H*), 4.64-4.68 (m, 2H, H-2' and Asn-α-C*H*), 6.13 (d, 1H, *J* = 6.7 Hz, H-1'), 7.54 (t, 1H, *J* = 8.3 Hz, H-6), 8.08 (s, 1H, C*H*O), 8.16 (d, 1H, *J* = 8.3 Hz, H-7), 8.26 (d, 1H, *J* = 8.3 Hz, H-5), 8.68 (s, 1H, H-2); ^{13}C NMR (150 MHz, D_2O) δ 8.9 (TEA-*C*H_3), 10.1 (Ile-δ-*C*H_3), 13.8 (Met-S*C*H_3), 14.7 (Ile-γ-*C*H_3), 16.1 (Ala-β-*C*H_3), 18.3 (Thr-γ-*C*H_3), 24.0 (Ile-γ-*C*H_2), 24.1 (Arg-γ-*C*H_2), 27.7 (Arg-β-*C*H_2), 28.7 (Met-γ-*C*H_2), 30.1 (Met-β-*C*H_2), 35.9 (Asn-β-*C*H_2), 36.6 (Ile-β-*C*H), 40.1 (Arg-δ-*C*H_2), 42.2 (Gly-α-*C*H_2), 45.6 (TEA-*C*H_2), 49.5 (Ala-α-*C*H), 49.9 (Asn-α-*C*H), 51.1 (Met-α-*C*H), 53.0 (Arg-α-*C*H), 58.6 (Thr-α-*C*H), 60.2 (Ile-α-*C*H), 66.9 (Thr-β-*C*H), 68.0 (C-5'), 70.0 (C-3'), 73.4 (C-2'), 82.8 (C-4'), 88.7 (C-1'), 119.0 (C-7), 120.5 (C-5), 123.2 (C-6), 134.8 (C-8), 135.9 (C-9), 137.7 (C-4), 145.4 (C-2), 156.2 (Arg-Cζ), 163.9 (*C*HO), 170.9 (Gly-CO), 171.8 (Asn-CO), 172.0 (Thr-CO), 173.0 (Met-CO), 173.3 (Arg-CO), 174.0 (Ala-CO and Asn-Cγ), 179.1 (Ile-CO); HRMS for $C_{43}H_{66}N_{15}O_{18}S_2$ ([M-H]$^-$) calcd: 1144.4158 found 1144.4150.

fMRTGNAI-SA (3.12b)

^{1}H NMR (600 MHz, D_2O) δ 0.85 (t, 3H, *J* = 7.2 Hz, Ile-δ-CH_3), 0.90 (d, 3H, *J* = 6.6 Hz, Ile-γ-CH_3), 1.13-1.24 (m, Thr-γ-CH_3, TEA-CH_3 and Ile-γ-CH_2, Ha), 1.36 (d, 3H, *J* = 7.2 Hz, Ala-β-CH_3), 1.43-1.52 (m, 1H, Ile-γ-CH_2, Hb), 1.61 (m, 2H, Arg-γ-CH_2), 1.71-1.92 (m, 3H, Ile β-C*H* and 2H, Arg-β-CH_2), 1.96-2.08 (m+s, 5H, Met-β-CH_2 and Met-SCH_3), 2.55 (m, 2H, Met-γ-CH_2), 2.70 (dd, 1H, *J* = 8.4 Hz and 15.6 Hz, Asn-β-CH_2, Ha), 2.79 (dd, 1H, *J* = 4.8 Hz, and 16.2 Hz, Asn-β-CH_2, Hb), 3.08-3.21 (m, TEA-CH_2 and Arg-δ-CH_2), 3.97 (d, 1H, *J* = 16.9 Hz, Gly-α-CH_2, Ha), 3.98 (d, 1H, *J* = 16.9 Hz, Gly-α-CH_2, Hb), 4.06 (d, 1H, *J* = 6.9 Hz, Ile-α-C*H*), 4.21 (m, 1H, Thr-β-C*H*), 4.26-4.46 (m, 7H, Ala-α-C*H*, Thr-α-C*H*, Arg-α-C*H*, H-3', H-4' and H-5'a and H-5'b), 4.50 (dd, 1H, *J* = 5.9 Hz, and 8.2 Hz, Met-α-C*H*), 4.67 (dd, 1H, *J* = 5.2 Hz and 8.9 Hz, Asn-α-C*H*), 4.72 (m, 1H, H-2'), 6.09 (d, 1H, *J* = 5.9 Hz, H-1'), 8.08 (s, 1H, C*H*O) , 8.22 (s, 1H, H-2), 8.42 (s, 1H, H-8); ^{13}C NMR (150 MHz, D_2O) δ 7.9 (TEA-*C*H_3), 9.8 (Ile-δ-*C*H_3), 13.8 (Met-S*C*H_3), 14.4 (Ile-γ-*C*H_3), 16.0 (Ala-β-*C*H_3), 18.4 (Thr-γ-*C*H_3), 24.1 (Ile-γ-*C*H_2), 24.3 (Arg-γ-*C*H_2), 27.6 (Arg-β-*C*H_2), 28.7 (Met-γ-*C*H_2), 30.1 (Met-β-*C*H_2), 35.4 (Asn-β-*C*H_2), 35.9 (Ile-β-*C*H), 40.1 (Arg-δ-*C*H_2), 42.1 (Gly-α-*C*H_2), 46.3 (TEA-*C*H_2), 49.5 (Ala-α-*C*H), 50.0 (Asn-α-*C*H), 51.1 (Met-α-*C*H), 53.2 (Arg-α-*C*H), 58.8 (Thr-α-*C*H), 60.4 (Ile-α-*C*H), 66.9 (Thr-β-*C*H), 68.0 (C-5'), 70.2 (C-3'), 73.7 (C-2'), 82.8 (C-4'), 86.5 (C-1'), 139.4 (C-8), 148.8 (C-4), 152.7 (C-2), 155.2 (C-6), 156.4 (Arg-Cζ), 163.9 (*C*HO), 170.9 (Gly-*C*O), 172.0 (Asn-*C*O and Thr-*C*O), 173.1 (Met-*C*O), 173.4 (Arg-*C*O), 174.0 (Ala-

*C*O and Asn-*C*γ), 179.6 (Ile-*C*O); HRMS for $C_{41}H_{68}N_{17}O_{16}S_2$ ([M-H]$^-$) calcd: 1116.4320 found 1116.4305.

fMRTGNAI-SG (3.12c)

^{1}H NMR (600 MHz, D_2O) δ 0.77 (t, 3H, *J* = 7.2 Hz, Ile-δ-C*H*$_3$), 0.82 (d, 3H, *J* = 6.6 Hz, Ile-γ-C*H*$_3$), 1.05 (m, 1H, Ile-γ-C*H*$_2$, Ha), 1.16 (d, *J* = 6.3 Hz, 3H, Thr-γ-C*H*$_3$), 1.24 (t, *J* = 3.6 Hz, TEA-C*H*$_3$), 1.32 (d, 3H, *J* = 7.2 Hz, Ala-β-C*H*$_3$), 1.36 (m, 1H, Ile-γ-C*H*$_2$, Hb), 1.58 (m, 2H, Arg-γ-C*H*$_2$), 1.69-1.89 (m, 3H, Ile β-C*H* and Arg-β-C*H*$_2$), 1.94-2.08 (m+s, 5H, Met-β-C*H*$_2$ and Met-SC*H*$_3$), 2.45-2.57 (m, 2H, Met-γ-C*H*$_2$), 2.70 (dd, 1H, *J* = 8.4 Hz and 15.6 Hz, Asn-β-C*H*$_2$, Ha), 2.80 (dd, 1H, *J* = 4.8 Hz and 16.2 Hz, Asn-β-C*H*$_2$, Hb), 3.11 (t, 2H, *J* = 6.6 Hz, Arg-δ-C*H*$_2$), 3.16 (q, *J* = 7.8 Hz, TEA-C*H*$_2$), 3.89 (d, 1H, *J* = 6.6 Hz and 16.6 Hz, Gly-α-C*H*$_2$, Ha), 3.95-4.02 (m, 2H, Ile-α-C*H* and Gly-α-C*H*$_2$, Hb), 4.18-4.24 (m, 1H, Thr-β-C*H*), 4.25-4.31 (m, 3H, H-5'a and H-5'b, Ala-α-C*H*), 4.33-4.37 (m, 2H, *J* = 4.4 Hz, Thr-α-C*H*, H-4'), 4.37-4.43 (m, 2H, Arg-α-C*H*, H-3'), 4.49 (dd, 1H, *J* = 5.9 Hz and 8.2 Hz, Met-α-C*H*), 4.66-4.72 (m, 2H, Asn-α-C*H* and H-2'), 5.89 (d, 1H, *J* = 6.6 Hz, H-1'), 8.00 (s, 1H, H-8), 8.08 (s, 1H, C*H*O); ^{13}C NMR (150 MHz, D_2O) δ 7.3 (TEA-*C*H$_3$), 10.0 (Ile-δ-*C*H$_3$), 13.6 (Met-S*C*H$_3$), 14.5 (Ile-γ-CH$_3$), 15.9 (Ala-β-*C*H$_3$), 18.2 (Thr-γ-*C*H$_3$), 23.8 (Ile-γ-*C*H$_2$), 23.9 (Arg-γ-*C*H$_2$), 27.7 (Arg-β-*C*H$_2$), 28.7 (Met-γ-*C*H$_2$), 29.8 (Met-β-*C*H$_2$), 35.8 (Asn-β-*C*H$_2$), 36.5 (Ile-β-*C*H), 40.1 (Arg-δ-*C*H$_2$), 42.1 (Gly-α-*C*H$_2$), 46.0 (TEA-*C*H$_2$), 49.5 (Ala-α-*C*H), 49.9 (Asn-α-*C*H), 51.1 (Met-α-*C*H), 53.0 (Arg-α-*C*H), 58.6 (Thr-α-*C*H), 60.2 (Ile-α-*C*H), 66.9 (Thr-β-*C*H), 68.1 (C-5'), 70.0 (C-3'), 73.1 (C-2'), 82.0 (C-4'), 85.9 (C-1'), 116.2 (C-5), 136.3 (C-8), 151.4 (C-4), 156.0 (Arg-Cζ), 163.7 (*C*HO), 170.6 (Gly-*C*O), 171.5 (Asn-*C*O), 171.7 (Thr-*C*O), 173.1 (Met-*C*O), 173.2 (Arg-*C*O), 173.7 (Ala-*C*O), 173.8 (Asn-*C*γ), 179.1 (Ile-*C*O); HRMS for $C_{41}H_{66}N_{17}O_{17}S_2$ ([M-H]$^-$) calcd: 1132.4269 found 1132.4274.

fMRTGNAI-SI (3.12d)

^{1}H NMR (600 MHz, D_2O) δ 0.75 (t, 3H, *J* = 7.2 Hz, Ile-δ-C*H*$_3$), 0.81 (d, 3H, *J* = 6.6 Hz, Ile-γ-C*H*$_3$), 1.04-1.10 (m, Ile-γ-C*H*$_2$, Ha, TEA-C*H*$_3$), 1.16 (d, 3H, *J* = 6.3 Hz, Thr-γ-C*H*$_3$),1.29-1.37 (d+m, 4H, *J* = 7.2 Hz, Ala-β-C*H*$_3$ and Ile-γ-C*H*$_2$, Ha), 1.55 (m, 2H, Arg-γ-C*H*$_2$), 1.69-1.77 (m, 2H, Ile-β-C*H* and Arg-β-C*H*$_2$, Ha), 1.79-1.87 (m, 1H, Arg-β-C*H*$_2$, Hb), 1.94-2.08 (m+s, 5H, Met-β-C*H*$_2$ and Met-SC*H*$_3$), 2.45-2.57 (m, 2H, Met-γ-C*H*$_2$), 2.67-2.76 (m, TEA-C*H*$_2$ and Asn-β-C*H*$_2$, Ha), 2.80 (dd, 1H, *J* = 4.8 Hz and 16.2 Hz, Asn-β-C*H*$_2$, Hb), 3.10 (t, 2H, *J* = 6.6 Hz, Arg-δ-C*H*$_2$), 3.87 (d, 1H, *J* = 16.8 Hz, Gly-α-C*H*$_2$, Ha), 3.95-4.01 (m, 2H, Ile-α-C*H* and Gly-α-C*H*$_2$, Hb), 4.18-4.24 (m, 1H, Thr-β-C*H*), 4.25-4.31 (m, 3H, H-5'a and H-5'b, Ala-α-C*H*), 4.33 (d, 1H, *J* = 4.4 Hz, Thr-α-C*H*), 4.36-4.43 (m, 3H, Arg-α-C*H*, H-3', H-4'), 4.48 (dd, 1H, *J* = 5.9 Hz and 8.2 Hz, Met-α-C*H*), 4.67-4.73 (m, 2H, Asn-α-C*H* and H-2'), 6.03 (d, 1H, *J* = 6.6 Hz, H-1'), 8.08 (s, 1H, C*H*O), 8.11 (s, 1H, H-2), 8.25 (s, 1H, H-8); ^{13}C NMR (150 MHz, D_2O)

δ 9.1 (TEA-*C*H_3), 10.1 (Ile-δ-*C*H_3), 13.8 (Met-S*C*H_3), 14.7 (Ile-γ-*C*H_3), 16.1 (Ala-β-*C*H_3), 18.4 (Thr-γ-*C*H_3), 24.2 (Ile-γ-*C*H_2 and Arg-γ-*C*H_2), 27.5 (Arg-β-*C*H_2), 28.8 (Met-γ-*C*H_2), 30.0 (Met-β-*C*H_2), 35.9 (Asn-β-*C*H_2), 36.6 (Ile-β-*C*H), 40.0 (Arg-δ-*C*H_2), 42.2 (Gly-α-*C*H_2), 45.5 (TEA-*C*H_2), 49.6 (Ala-α-*C*H), 50.0 (Asn-α-*C*H), 51.3 (Met-α-*C*H), 53.2 (Arg-α-*C*H), 58.8 (Thr-α-*C*H), 60.4 (Ile-α-*C*H), 66.9 (Thr-β-*C*H), 68.2 (C-5'), 70.3 (C-3'), 73.5 (C-2'), 82.2 (C-4'), 86.1 (C-1'), 123.0 (C-5), 137.3 (C-8), 149.8 (C-4), 153.6 (C-2), 156.2 (Arg-*C*ζ), 164.0 (*C*HO), 167.3 (C-6), 170.9 (Gly-*C*O), 171.8 (Asn-*C*O), 172.1 (Thr-*C*O), 173.3 (Met-*C*O), 173.6 (Arg-*C*O), 174.0 (Ala-*C*O), 174.1 (Asn-*C*γ), 179.4 (Ile-*C*O); HRMS for $C_{41}H_{65}N_{16}O_{17}S_2$ ([M-H]$^-$) calcd: 1117.4160 found 1117.4186.

fMRTGNAI-SC (3.12e)

^{1}H NMR (600 MHz, D_2O) δ 0.82 (t, 3H, *J* = 7.2 Hz, Ile-δ-C*H*$_3$), 0.88 (d, 3H, *J* = 6.6 Hz, Ile-γ-C*H*$_3$), 1.07-1.15 (m, 1H, Ile-γ-C*H*$_2$, Ha), 1.15-1.24 (m, Thr-γ-C*H*$_3$ and TEA-C*H*$_3$), 1.35 (d, 3H, *J* = 7.2 Hz, Ala-β-C*H*$_3$), 1.37-1.44 (m, 1H, Ile-γ-C*H*$_2$, Hb), 1.61 (m, 2H, Arg-γ-C*H*$_2$), 1.75 (m, 1H, Ile-β-C*H*), 1.84 (m, 2H, Arg-β-C*H*$_2$), 1.96-2.09 (m+s, 5H, Met-β-C*H*$_2$ and Met-SC*H*$_3$), 2.55 (m, 2H, Met-γ-C*H*$_2$), 2.70 (m, 1H, Asn-β-C*H*$_2$, Ha) , 2.80 (dd, 1H, *J* = 4.8 Hz and 16.2 Hz, Asn-β-C*H*$_2$, Hb), 3.07 (m, TEA-C*H*$_2$), 3.16 (t, 2H, *J* = 6.6 Hz, Arg-δ-C*H*$_2$), 3.89 (d, 1H, *J* = 5.4 Hz, Gly-α-C*H*$_2$, Ha), 3.95-4.04 (m, 2H, Ile-α-C*H* and Gly-α-C*H*$_2$, Hb), 4.18-4.25 (m, 2H, H-2' and Thr-β-C*H*), 4.27-4.33 (m, 4H, H-5'a, H-5'b, H-3' and Ala-α-C*H*), 4.34-4.45 (m, 3H, Thr-α-C*H*, Arg-α-C*H* and H-4'), 4.50 (dd, 1H, *J* = 5.9 Hz and 8.2 Hz, Met-α-C*H*), 4.68 (dd, 1H, *J* = 5.2 Hz and 8.9 Hz, Asn-α-C*H*), 5.92 (d, 1H, *J* = 3.2 Hz, H-1'), 6.03 (d, 1H, *J* = 7.5 Hz, H-5), 7.82 (d, 1H, *J* = 7.5 Hz, H-6), 8.09 (s, 1H, C*H*O); ^{13}C NMR (150 MHz, D_2O) δ 8.3 (TEA-*C*H_3), 10.3 (Ile-δ-*C*H_3), 13.8 (Met-S*C*H_3), 14.8 (Ile-γ-*C*H_3), 16.2 (Ala-β-*C*H_3), 18.4 (Thr-γ-*C*H_3), 24.2 (Ile-γ-*C*H_2 and Arg-γ-*C*H_2), 27.5 (Arg-β-*C*H_2), 28.8 (Met-γ-*C*H_2), 30.0 (Met-β-*C*H_2), 35.9 (Asn-β-*C*H_2), 36.7 (Ile-β-*C*H), 40.1 (Arg-δ-*C*H_2), 42.2 (Gly-α-*C*H_2), 46.0 (TEA-*C*H_2), 49.6 (Ala-α-*C*H), 50.0 (Asn-α-*C*H), 51.3 (Met-α-*C*H), 53.2 (Arg-α-*C*H), 58.8 (Thr-α-*C*H), 60.4 (Ile-α-*C*H), 66.9 (Thr-β-*C*H), 67.2 (C-5'), 69.0 (C-3'), 73.8 (C-2'), 81.1 (C-4'), 89.1 (C-1'), 96.2 (C-5), 140.9 (C-6), 156.3 (Arg-*C*ζ), 157.4 (C-4), 164.0 (*C*HO), 165.8 (C-2), 170.9 (Gly-*C*O), 171.9 (Asn-*C*O), 172.1 (Thr-*C*O), 173.4 (Met-*C*O), 173.6 (Arg-*C*O), 174.1 (Ala-*C*O and Asn-*C*γ), 179.4 (Ile-*C*O); HRMS for $C_{40}H_{66}N_{15}O_{17}S_2$ ([M-H]$^-$) calcd: 1092.4208 found 1092.4196.

fMRTGNAI-SU (3.12f)

^{1}H NMR (600 MHz, D_2O) δ 0.83 (t, 3H, *J* = 7.2 Hz, Ile-δ-C*H*$_3$), 0.89 (d, 3H, *J* = 6.6 Hz, Ile-γ-C*H*$_3$), 1.07-1.16 (m, 1H, Ile-γ-C*H*$_2$, Ha), 1.18 (d, 3H, *J* = 8.4 Hz, Thr-γ-C*H*$_3$), 1.19-1.27 (t, *J* = 3.6 Hz, TEA-C*H*$_3$), 1.36 (d, 3H, *J* = 7.2 Hz, Ala-β-C*H*$_3$), 1.37-1.44 (m, 1H, Ile-γ-C*H*$_2$, Hb), 1.61 (m, 2H, Arg-γ-C*H*$_2$), 1.7-1.92 (m, 3H, Ile-β-C*H* and 2H, Arg-β-C*H*$_2$), 1.98-2.09 (m+s, 5H, Met-β-C*H*$_2$ and Met-SC*H*$_3$), 2.55 (m, 2H, Met-γ-C*H*$_2$), 2.70

(dd, 1H, *J* = 8.4 Hz and 15.6 Hz, Asn-β-CH_2, Ha), 2.80 (dd, 1H, *J* = 4.8 Hz, and 16.2 Hz, Asn-β-CH_2, Hb), 3.17 (m, TEA-CH_2 and Arg-δ-CH_2), 3.89 (d, 1H, *J* = 16.9 Hz, Gly-α-CH_2, Ha), 3.98 (d, 1H, *J* = 16.9 Hz, Gly-α-CH_2, Hb), 4.01 (d, 1H, *J* = 6.9 Hz, Ile-α-C*H*), 4.19-4.37 (m, 8H, Thr-β-C*H*, Ala-α-C*H*, Thr-α-C*H*, Arg-α-C*H*, H-3', H-4', H-5'a-and H-5'b), 4.41 (dd, 1H, *J* = 5.9 Hz and 8.2 Hz, Met-α-C*H*), 4.51 (dd, 1H, *J* = 6.0 Hz, and 8.3 Hz, H-2'), 4.68 (dd, 1H, *J* = 5.2 Hz and 8.9 Hz, Asn-α-C*H*), 5.88 (d, 1H, *J* = 7.6 Hz, H-5), 5.94 (d, 1H, *J* = 5.3 Hz, H-1'), 7.79 (d, 1H, *J* = 7.6 Hz, H-6), 8.09 (s, 1H, C*H*O); ^{13}C NMR (150 MHz, D_2O) δ 7.9 (TEA-*C*H_3), 10.2 (Ile-δ-*C*H_3), 13.8 (Met-S*C*H_3), 14.8 (Ile-γ-*C*H_3), 16.2 (Ala-β-*C*H_3), 18.4 (Thr-γ-*C*H_3), 24.2 (Ile-γ-*C*H_2), 24.3 (Arg-γ-*C*H_2), 27.6 (Arg-β-*C*H_2), 28.8 (Met-γ-*C*H_2), 30.0 (Met-β-*C*H_2), 35.9 (Asn-β-*C*H_2), 36.6 (Ile-β-*C*H), 40.1 (Arg-δ-*C*H_2), 42.2 (Gly-α-*C*H_2), 46.3 (TEA-*C*H_2), 49.5 (Ala-α-*C*H), 50.0 (Asn-α-*C*H), 51.3 (Met-α-*C*H), 53.2 (Arg-α-*C*H), 58.8 (Thr-α-*C*H), 60.4 (Ile-α-*C*H), 66.8 (Thr-β-*C*H), 67.6 (C-5'), 69.4 (C-3'), 73.4 (C-2'), 81.5 (C-4'), 88.2 (C-1'), 102.4 (C-5), 140.8 (C-6), 156.3 (Arg-*C*ζ), 152.8 (C-2), 164.0 (*C*HO), 167.4 (C-4), 170.9 (Gly-*C*O), 171.9 (Asn-*C*O), 172.1 (Thr-*C*O), 173.3 (Met-*C*O), 173.6 (Arg-*C*O), 174.0 (Ala-*C*O and Asn-*C*γ), 179.5 (Ile-*C*O); HRMS for $C_{40}H_{65}N_{14}O_{18}S_2$ ($[M-H]^-$) calcd: 1093.4048 found 1093.4047.

fMRTGNAI-S(4-ABI) (3.12g)

^{1}H NMR (600 MHz, D_2O) δ 0.76 (t, 3H, *J* = 7.2 Hz, Ile-δ-CH_3), 0.84 (d, 3H, *J* = 6.6 Hz, Ile-γ-CH_3), 1.05-1.11 (m, 1H, Ile-γ-CH_2, Ha), 1.16 (d, 4H, *J* = 6.3 Hz, 3H, Thr-γ-CH_3), 1.22 (t, *J* = 3.6 Hz, TEA-CH_3), 1.30 (d, 3H, *J* = 7.2 Hz, Ala-β-CH_3), 1.36-1.40 (m, 1H, Ile-γ-CH_2, Hb), 1.51-1.60 (m, 2H, Arg-γ-CH_2), 1.60-1.72 (m, 1H, Ile-β-C*H*), 1.77-1.85 (m, 2H, Arg-β-CH_2), 1.94-2.08 (m+s, 5H, Met-β-CH_2 and Met-SCH_3), 2.47-2.58 (m, 2H, Met-γ-CH_2), 2.97 (dd, 1H, *J* = 8.4 Hz and 15.6 Hz, Asn-β-CH_2, Ha), 2.78 (dd, 1H, *J* = 4.8 Hz, and 16.2 Hz, Asn-β-CH_2, Hb), 3.05-3.15 (m, Arg-δ-CH_2 and TEA-CH_2), 3.87 (dd, 1H, *J* = 6.6 Hz and 16.6 Hz, Gly-α-CH_2, Ha), 3.94-4.03 (m, 2H, Ile-α-C*H* and Gly-α-CH_2, Hb), 4.17-4.23 (m, 1H, Thr-β-C*H*), 4.23-4.29 (m, 1H, Ala-α-C*H*), 4.30-4.43 (m, 6H, Thr-α-C*H*, Arg-α-C*H*, H-3', H-4', H-5'a and H-5'b), 4.48 (t, 1H, *J* = 7.2 Hz, Met-α-C*H*), 4.64-4.68 (m, 2H, Asn-α-C*H* and H-2'), 5.99 (d, 1H, *J* = 6.0 Hz, H-1'), 6.73 (d, 1H, *J* = 7.2 Hz, H-5), 7.15-7.21 (d+tr, 2H, H-6, and H-7), 8.01 (s, 1H, C*H*O), 8.31 (s, 1H, H-2); ^{13}C NMR (150 MHz, D_2O) δ 8.0 (TEA-*C*H_3), 10.1 (Ile-δ-*C*H_3), 13.8 (Met-S*C*H_3), 14.8 (Ile-γ-*C*H_3), 16.1 (Ala-β-*C*H_3), 18.4 (Thr-γ-*C*H_3), 24.2 (Ile-γ-*C*H_2 and Arg-γ-*C*H_2), 27.5 (Arg-β-*C*H_2), 28.8 (Met-γ-*C*H_2), 30.0 (Met-β-*C*H_2), 35.9 (Asn-β-*C*H_2), 36.6 (Ile-β-*C*H), 40.0 (Arg-δ-*C*H_2), 42.2 (Gly-α-*C*H_2), 46.2 (TEA-*C*H_2), 49.6 (Ala-α-*C*H), 49.9 (Asn-α-*C*H), 51.3 (Met-α-*C*H), 53.2 (Arg-α-*C*H), 58.7 (Thr-α-*C*H), 60.4 (Ile-α-*C*H), 66.9 (Thr-β-*C*H), 68.1 (C-5'), 70.0 (C-3'), 72.6 (C-2'), 82.3 (C-4'), 88.2 (C-1'), 102.1 (C-7), 108.5 (C-5), 124.8 (C-6), 132.2 (C-9), 133.0 (C-8) 137.4 (C-4), 140.7 (C-2), 156.2 (Arg-*C*ζ), 164.0 (*C*HO), 170.9 (Gly-*C*O), 171.8 (Asn-*C*O), 172.1 (Thr-*C*O), 173.3 (Met-

*C*O), 173.6 (Arg-*C*O), 174.0 (Ala-*C*O), 174.1 (Asn-*C*γ), 179.4 (Ile-*C*O); HRMS for $C_{43}H_{68}N_{15}O_{16}S_2$ ([M-H]$^-$) calcd: 1114.4415 found 1114.4412.

3.6.2 Model building and analysis

With the *E coli* Ile-tRNA synthetase structure being not available in the protein databank, a homology model was created using the I-tasser server.[130] The sequence of the enzyme was taken from the uniprot database (enzyme code EC=6.1.1.5 and uniprot id P00956). As single 3D template structure, we used the *E coli* methionyl-tRNA synthetase structure (1PG0) belonging to the same class I of tRNA synthetases. This structure contains a methionyl adenylate analogue inhibitor similar to the seven inhibitors used in this study, and therefore was used as template for our adenylate inhibitor. Additionally, the threonyl sulfamoyl adenosine inhibitor found in structure 3UH0[138] was used to complete part of the sulfamoyl tail in our inhibitor structures. The six inhibitors having different bases were built by superimposing the respective base onto the adenosine using quatfit. The homology model of *E coli* Ile-tRNA synthetase was then superimposed onto structure 1PG0 using dali.[139]

All seven inhibitors were then superimposed onto the original inhibitor present in 1PG0 resulting in seven complexes of *E coli* Ile-tRNA synthetase with inhibitor. As no interactions were seen between the base atoms of the pyridine inhibitors and the enzyme pocket, the uridine and cytosine inhibitors were repositioned with the base coinciding with the six-membered ring of the adenine base. The complexes were prepared for use in the AMBER software.[140] The parameters for enzyme structure were taken from the ff99bsc0 force field.[141] The seven ligand molecules were parameterized with Antechamber using the gaff force field.[142] Some patches were introduced to the parameters to get the right conformation for the base atoms and the sulfamoyl group after energy minimization. The molecular mechanics energy of the complexes was minimized using 500 steps. Then the mm/pbsa method[143] was applied on the minimized complexes to obtain the binding energies for the different inhibitors. Visual inspection[144] together with a ligplot analysis[145] was used for interpretation.

3.6.3 Biological activity experiments

3.6.3.1 Whole cell activity determinations

The respective bacteria were grown overnight in Luria Broth (LB) medium and cultured again the following day in fresh LB medium or LB-medium containing 5 mM (L)-arabinose. Compounds were titrated in a 96-well plate using either LB-medium +/- 5 mM (L)-arabinose to dilute the compounds. To each well, 85 µL LB-medium +/- 5 mM (L)-arabinose was added to a total volume of 90 µL. Next, 10 µL of bacterial cell culture grown to a OD_{600} of 0.1 was added. The cultures were next placed into a Tecan Infinite M200® incubator and shaken at 37 °C, subsequently the OD_{600} was determined after 18 h. The broth dilution tests were performed in duplicates.

Bacterial strains used for the evaluations: *E. coli* Ara-Yej (BW39758), expressing the YejABEF transporter upon L-arabinose induction; *E. coli* wt used as wild type control. The antibacterial activities of all compounds were determined by monitoring the optical density of suspensions of cell-cultures.

3.6.3.2 Aminoacylation experiments

To assess the degree of inhibition of the aminoacylation reaction, *in vitro* tests were perform using the relevant S30 cell extracts.

Preparation of S30 cell extracts. Cells were grown in 50 mL LB-medium. After centrifugation at 3000 g for 10 min the supernatant was discarded and the pellet was resuspended in 40 mL buffer containing: Tris.HCl or Hepes.KOH (pH = 8.0; 20 mM), $MgCl_2$ (10 mM), KCl (100 mM). The cell suspension was centrifuged again at 3000 g. This procedure was repeated twice. The pellet was resuspended in 1 mL of the following buffer Tris.HCl or Hepes.KOH (pH = 8.0; 20 mM), $MgCl_2$ (10 mM), KCl (100 mM), DTT (1 mM) and kept at 0 °C. Subsequently, the cells were sonicated for 10 s and left at 0 °C for 10 min. This procedure was repeated 5-8 times. The lysate was centrifuged at 15000 g for 30 min at +4 °C.

tRNA aminoacylation reaction: To 1 μL of solution containing inhibitor, 3 μL of *E. coli* S30 extracts was added. Next, 16 μL of the following aminoacylation mixture was added: Tris.HCl (30 mM, pH 8.0), DTT (1 mM), bulk of *E. coli* tRNA (5 g/l), ATP (3 mM), KCl (30 mM), $MgCl_2$ (8 mM), and the specified, ^{14}C-radiolabeled amino acid (40 μM, 200 μCi/mmol). The reaction products were precipitated in cold 10% TCA on Whatman 3MM papers, 5 min. after the aminoacylation mixture was added. The aminoacylation reaction was carried out at room temperature. Depending on whether or not processing was needed, variable time intervals were included between the addition of the cell extract and the addition of the aminoacylation mixture. After thorough washing with cold 10% TCA, the papers were washed twice with acetone and dried on a heating plate. Following the addition of scintillation liquid (12 mL), the amount of radioactivity was determined in a Tri-card 2300 TR (time resolved) liquid scintillation counter. ^{14}C-Radiolabeled amino acids and scintillation liquid were purchased from Perkin Elmer.

4 5'-(*N*-aminoacyl)-sulfonamido-5'-deoxyadenosine: attempts for a stable alternative to aminoacyl-sulfamoyl adenosines

Abstract

Upon synthesis of aminoacyl-sulfamoyl adenosines (aaSAs) and their peptidyl conjugates, we observed that aminoacyl-sulfamoyl adenosines are prone to form a cycloadenosine derivative by attack of the adenine N^3 on the C-5'of the ribose. In an effort to reduce this side reaction, we aimed to prepare aminoacyl-sulfonamide (aa-SoA) as a more stable alternative to aaSA derivatives. We hypothesized that deletion of the 5'-oxygen in aaSA analogues renders the C-5' less electrophilic which in turn should improve the stability in comparison to aminoacyl sulfamate analogues. We therefore synthesized six aa-sulfonamides and further compared their activity against the respective aaSA analogue. It was shown however that these compounds are not able to inhibit the corresponding aaRS, except for the aspartyl derivative. We in addition tested the intermediate sulfamate (SA) and sulfonamide (SoA) (without any attached amino acid) for inhibitory activity. The obtained results will be discussed in comparison with their corresponding aaSA analogues.

4.1 Introduction

Despite of numerous aaRS inhibitors reported in the literature, none of them (except mupirocin) developed into a clinically useful antibiotic.[146] Therefore, in recent years, more efforts have been directed towards the rational design of aaRS inhibitors based on the natural reaction intermediate (aa-AMP).[16] In fact, the idea to explore non-hydrolysable analogues of aa-AMP is also derived from natural antibiotics and examples include microcin C (**1.17**),[78, 80, 82] agrocin 84 (**1.14**)[54b, 54c] and ascamycin (**1.15a**)[55] (Figure 1-5). Here, microcin C and agrocin 84 possess an acyl-phosphoramidate linkage as a replacement for the labile acyl-phosphate whereas ascamycin comprises a stable sulfamate linkage. These antibiotics have been discussed in section 1.3.1 (Chapter 1). Among several non-hydrolysable mimics of aa-AMP, aaSA analogues are proved to be the strongest inhibitor of the corresponding aaRS *in vitro*. However, they could be not pursued as potential antibiotics due to their lack of selectivity and the poor *in vivo* efficacy. Moreover, synthesis of aaSA analogues remained problematic due to the cyclic degradation formed during synthesis. It has been reported that the aaSAs are prone to form a cycloadenosine derivative as a side product. This degradation results in poor yield of the reaction.[114] In our attempts to promote the uptake of aaSA analogues, we consistently observed the formation of cycloadenosine as a side product.[83] Similar problems were also encountered by Van de Vijver *et al.* and Vodenhoff G. *et al.* while studying dipeptidyl- and hexapeptidyl-conjugates of sulfamoyl adenosines respectively.[81, 114b, 147] Therefore, in this chapter we investigated aaSoA as a potentially more stable alternative to aaSA analogues. We hypothesized that eliminating the 5'-oxygen of aaSA may render the C-5' of ribose less susceptible to electrophilic attack by N3 of the adenine, and thus could yield more stable analogues which hopefully retain the inhibitory activity with equal potency as compared to aaSA analogues. General structures of an aaSA (**4.1**) analogue along with the proposed aa-sulfonamide (**4.2a**) have been given in Figure 4-1.

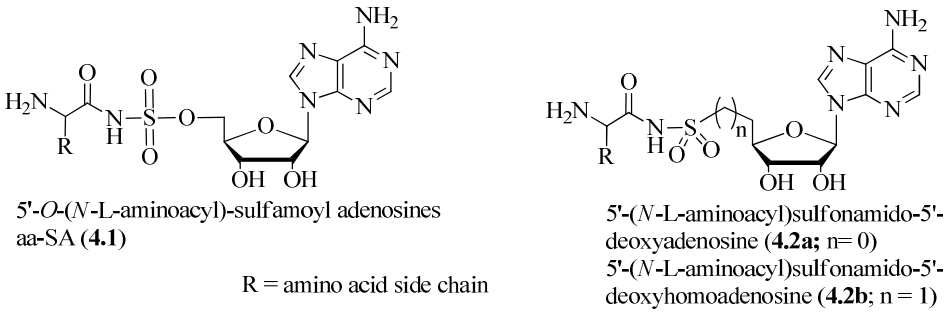


Figure 4-1: General structures of aaSA analogue and proposed aaSoA analogue.

4.2 Design

As mentioned above, among the different non-hydrolysable mimics of aa-AMP, aaSAs proved to be excellent inhibitors of the corresponding aaRS *in vitro*. However, these analogues could not be pursued further due to their lack of selectivity and poor cell penetration. Several modifications have been attempted to address these issues. For example, a series of aryl-tetrazole containing sulfamate derivatives reported by Cubist Pharmaceuticals showed 3000-fold selectivity for pathogen aaRS over human aaRS. Despite their high selectivity and excellent inhibitory potency *in vitro*, these analogues could not reach the clinic due to their high serum albumin binding and poor cell penetration.[8, 61] In chapter 1, we attempted to improve the *in vivo* efficacy of aryl-tetrazole containing sulfamates by coupling them with a trihydroxamate siderophore (iron carrier) but without success.[83] Along the same lines, we also coupled aryl-tetrazole containing sulfamates with the McC hexapeptide (a transport module) in an attempt to improve their *in vivo* efficacy by a Trojan-horse mechanism.[83] Unexpectedly, both these conjugates (either siderophore or McC hexapeptide) failed to cross the cell membrane. We therefore concluded that the adenine base may be playing a vital role in recognition by the transporter (being either an iron channel or the YejABEF peptide transporter). Hence, the conjugates of different aminoacyl-sulfamoyl adenosines would seem interesting target compounds, as they should be recognized by the transporter and once internalized could act by a Trojan-horse mechanism. However, trihydroxamate-aaSA conjugates could not be synthesized due to their instability (Scheme 2-4, chapter 2). Moreover, synthesis of McC hexapeptidyl-aaSA conjugates is low yielding in part as of formation of a cyclic degradation product by the nucleophilic attack of the adenine N^3 on the sugar 5'-carbon.[81, 83] Similar problems were encountered by Van de Vijver *et al.*

when studying dipeptidyl-sulfamoyladenosine as potential antibiotics,[147] and aaSA analogues as immunosuppressant[114b] and by Vondenhoff *et al.* while synthesizing hexapeptidyl conjugates of aaSA.[81] We therefore aimed to improve the stability of the aaSA analogues and hypothesized that deletion of the 5'-oxygen of adenosine would render the C-5' less electrophilic and less prone to attack by N^3 of adenine. In general, sulfonamides are known to exhibit enzyme inhibitory activity in view of their non-hydrolysable properties.[148] Therefore, deletion of the 5'-oxygen could yield aaSoAs (**4.2a**) with improved stability and hopefully equal potency as compared to aaSAs.

To test our hypothesis, six aaSoAs **4.21-4.26** were synthesized and evaluated for their ability to inhibit the corresponding aaRS. Herein, sulfonamides **4.21-4.23** are targeting IleRS, LeuRS and TyrRS respectively belonging to class I of the synthetases, whereas sulfonamides **4.24-4.26** are targeting GlyRS, SerRS and AspRS respectively from class II of the synthetases. Isoleucyl- (**4.21**) and leucyl- (**4.22**) sulfonamides were selected for their straightforward synthesis while tyrosyl sulfonamide (**4.23**) was selected for its aromatic side chain. All three aaRSs have been targeted in the past of different medicinal chemistry efforts for inhibition of various microorganisms. Glycyl-sulfonamide **4.24** was selected for its small size and specifically for the considerable sequence divergence of the hetero-tetramer structure as found in eubacteria compared to the dimeric structure of human GlyRS.[149] Seryl-(**4.25**) and Aspartyl- (**4.26**) sulfonamide were selected for their polar side chain and as they already figured as targets in our previous efforts for developing antibiotics based on either microcin C or albomycin.[83] In addition, the intermediates in the synthesis of aaSoA (sulfonamide **4.27**) and aaSAs (sulfamate **4.29**) which lack an attached amino acid were also evaluated for their inhibitory properties.

4.3 Chemistry

Retro synthetic analysis of aa-sulfonamide reveals that the target aa-sulfonamide (**4.2a**) can be synthesized starting from an appropriately protected 5'-sulfonic acid derivative (**4.5**) via 5'-sulfonamido-5'-deoxyadenosine (**4.3**) (Figure 4-2). Active ester mediated coupling of **4.3** with an appropriately protected amino acid (**4.4**) could yield the desired aminoacyl-sulfamoyl adenosines (**4.2a**). Therefore, initial efforts have been directed towards synthesis of derivative **4.3**.

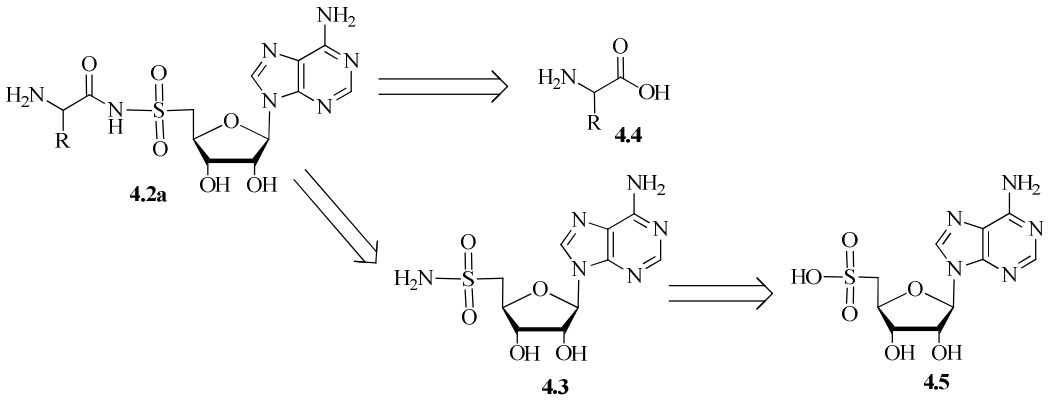
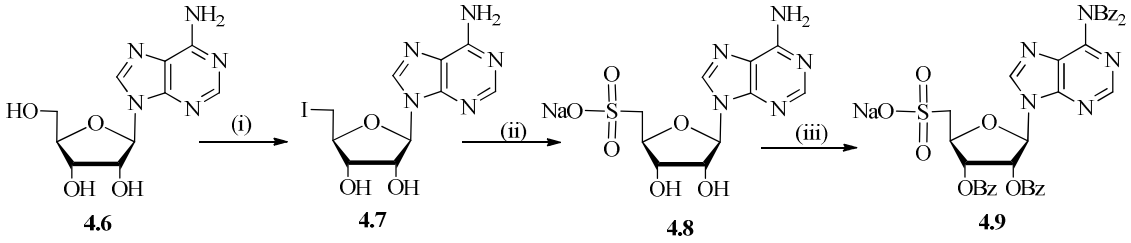
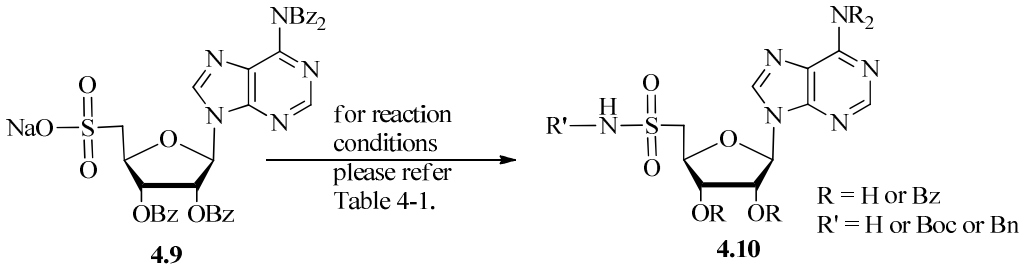


Figure 4-2: Retrosynthetic analysis of aminoacyl-sulfonamide (**4.2a**).

As shown in Scheme 4-1, under Mitsunobu reaction conditions, the 5'-hydroxyl of adenosine (**4.6**) was converted to a good leaving group (iodo derivative, **4.7**) which upon nucleophilic substitution using sodium sulphite (Na_2SO_3) gave sodium-5'-deoxyadenosine-5'-sulfonate (**4.8**). Further benzoylation of the compound **4.8** using pyridine as a base and DMF as a solvent gave compound **4.9**. However, converting 5'-sulfonate into a 5'-sulfonamide **4.10** proved to be cumbersome (Scheme 4-2). Our initial unsuccessful attempts for converting sulfonate **4.9** into sulfonamide are summarized in Table 4-1.



Scheme 4-1: Synthesis of protected sodium-5'-deoxy-adenosine-5'-sulfonate.
Reagents and conditions: (i) PPh_3, I_2, pyridine, rt, 2 h, (ii) Na_2SO_3, water: methanol, reflux, 18 h; (iii) BzCl, pyridine, DMAP, DMF, rt, 24 h.



Scheme 4-2: Attempted synthetic strategy in obtaining the 5'-sulfonamide scaffold.

Table 4-1: Summary of unsuccessful attempts to convert sodium sulfonate into sulfonamide

Entry	Reaction conditions	R'	R	
1.	1. $SOCl_2$ (1.5 eq.), 2 drops of DMF, Toluene, 0^o C addition, reflux for 18 h, 2. methanolic NH_3 0^o C to rt 24 h	H	H	Reaction mixture turned black upon heating (degradation)
2.	1. $SOCl_2$ (2 eq.), 3 drops of DMF, DCM, rt for 18 h 2. Methanolic ammonia, 0^o C to rt, 24 h	H	H	After 1st step, MS for SM-Bz was observed Degradation
3.	1. $SOCl_2$ (1.5 eq.), 3 drops of DMF, DCM, rt for 20 h 2. Boc carbamate, TEA, DCM	Boc	Bz	MS for SM-Bz was observed No reaction
4.	1. $SOCl_2$ as solvent, 40^o C, 20h Evaporation under reduced press Then dissolved in dry DCM 2. methanolic ammonia, 0^o C to rt 2 d	H	H	Degradation
5.	1. SO_2Cl_2, PPh_3, DCM, 0^o C to rt, 4h 2. $BnNH_2$, TEA, 0^o C to rt 4h	$PhCH_2$-	Bz	Degradation
6.	1.Sulfuryl chloride (5 eq.), 15-crown-5 ether (0.2 eq.), dry DCM 0^o C to rt, 4h, 2. TEA, $BnNH_2$	$PhCH_2$-	Bz	Degradation
7.	1. $SOCl_2$ (4 eq), 15-crown-5 (0.2 eq), 1 drop of DMF, DCM, 0°C addition, react for 1h at 0°C, bring to rt and react overnight, 2. Benzylamine (1.5 eq), triethylamine (7.5 eq), DCM, 0°C addition, react for 1h at 0°C and 1h at rt	$PhCH_2$-	Bz	Degradation
8.	Cyanuric chloride (1 eq.), 15-crown-5 ether (0.2 eq.), TEA (1 eq.), dry aetone reflux 20 h,Solvent was evaporated, and residue was dissolved in dry DCM and added to methanolic ammonia	H	H	Degradation
9.	1. Oxalyl chloride (1eq.), DCM, 5-15^o C, 1h, 2. TEA, $BnNH_2$, addition at 0^o C and 2 h at rt	$PhCH_2$-	Bz	No reaction
10.	1. PPh_3, Br_2, CH_3CN 2. $BnNH_2$, TEA	$PhCH_2$-	Bz	Degradation
11.	1. triphosgene, dry DCM 2. methanolic ammonia	H	H	Degradation

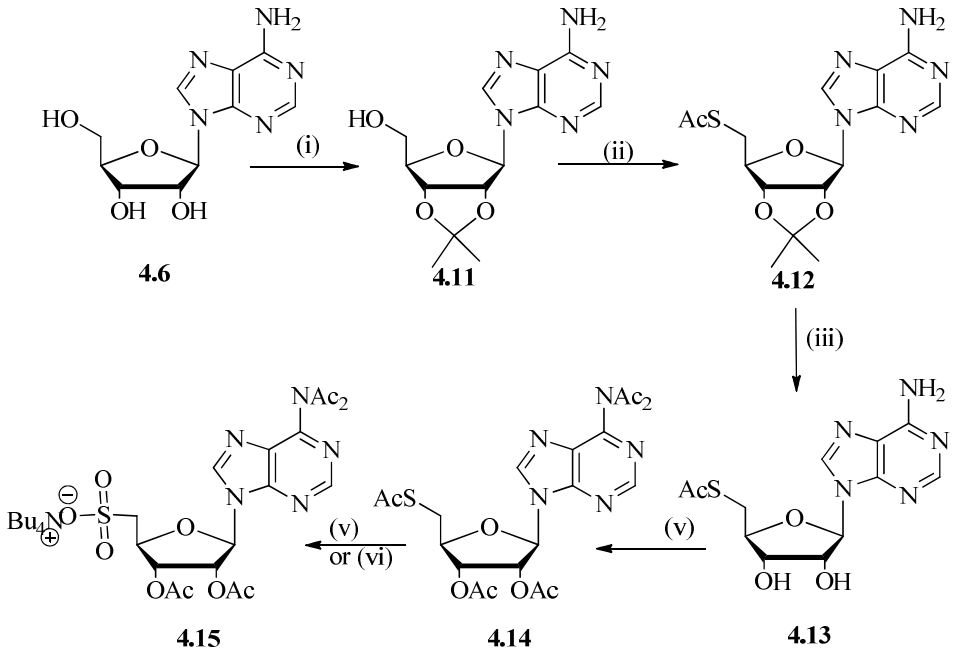
Thionyl chloride being widely used as a chlorinating agent was attempted to activate the sodium sulfonate **4.9** into sulfonyl chloride,[150] which upon reaction with ammonia could afford the desired sulfonamide. Optimization of the reaction conditions was attempted by changing the solvent, the number of equivalents of thionyl chloride, the reaction temperature (0^o C, 40^o C and reflux conditions) and the nucleophile (ammonia, Boc-carbamate or benzylamine). However, none of these efforts yielded the desired

sulfonamide (entry 1 to 4, Table 4-1) and rather degradation of the starting material was observed at higher equivalents of thionyl chloride or at higher temperature. Therefore, sulfuryl chloride,[151] cyanuric chloride[152] or oxalyl chloride were used as mild chlorinating agents (entry 5, 7 to 9). Unfortunately, these reagents likewise led to degradation of the starting sulfonate. It was clear from these experiments that the low nucleophilic reactivity of the sodium sulfonatewas at the heart of the problem for this failure. Therefore, we added 15-crown-5 ether to complex sodium and to increase the nucleophilicity of the sulfonate. Despite using crown ether, we again failed to obtain the desired sulfonamide using either sulfuryl chloride or thionyl chloride (entry 6 and 7 respectively) as a chlorinating agent. We therefore chose to use harsh reaction conditions such as triphenylphosphine with liquid bromine[153] or triphosgene[150b] (entry 10 and 11 respectively) with the hope to activate the sodium sulfonate into sulfonyl chloride but without success.

All of the above attempts failing to convert the sodium sulfonate into the desired sulfonamide, we decided to first prepare the tetrabutyl ammonium (TBA) salt instead. Previously, analogous tetrabutyl ammonium sulfonates have been successfully converted into a sulfonamide.[154] Synthesis of the tetrabutyl ammonium sulfonate is depicted in Scheme 4-3. As shown, adenosine (**4.6**) was first protected by an isopropylidene moiety to afford compound **4.11**. Next, the acetylated 5'-thio-5'-deoxyadenosine (**4.12**) was synthesized in almost quantitative yield by coupling thioacetic acid via a Mitsunobu reaction.[155] The isopropylidene protecting group was next removed by treatment with aqueous formic acid to afford compound **4.13**. Further, 2',3'-hydroxyl and 6-amino groups were protected as acetates. However, attempted oxidation of the protected thioacetyl derivative **4.14** to the tetrabutyl ammonium sulfonate **4.15** yielded over oxidized product with concomitant N^6-oxidation even with a milder oxidizing agent like oxone (potassium peroxymonosulfate).[151b, 156] Moreover, purification of the desired product remained challenging due to partial or complete deprotection of the acetyl group during the oxidation step.

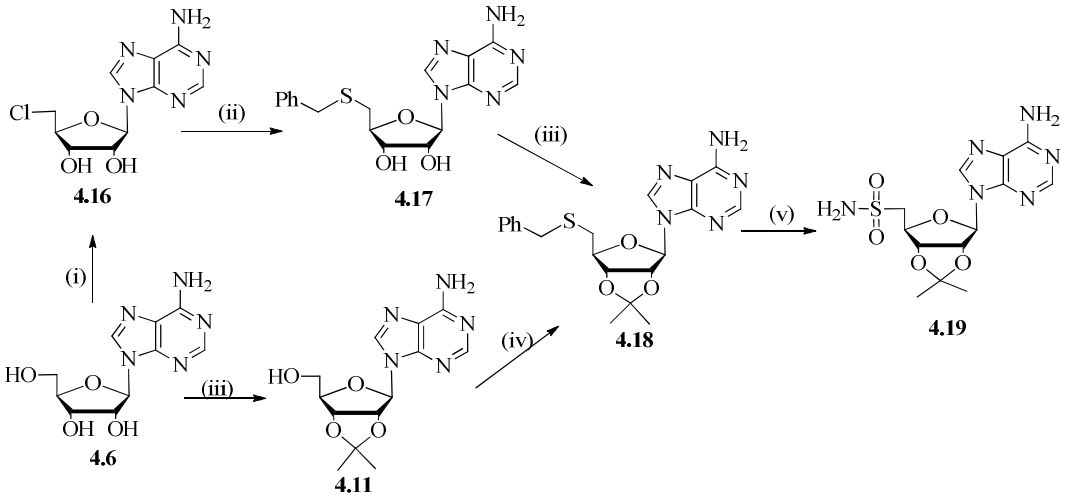
We therefore needed to look for an alternative strategy. It has been recently reported in the literature that aryl benzyl thioether or aryl thiols can be converted to the corresponding sulfonyl chlorides via oxidative chlorination using 1,3-dichloro-5,5-dimethyl hydantoin (DCDMH).[157] We attempted a similar protocol on the corresponding

nucleoside benzyl thioether (**4.17**) as well as on the thiol **4.20**. Nucleoside 5'-benzyl thioether was prepared via 5'-chloroadenosine derivative (**4.16**)[158] or directly using a Mitsunobu reaction (Scheme 4-4) on adenosine as per literature procedure.[155] In both cases, the yield for the reaction to obtain our starting benzyl thioether **4.17** was very low (<40%). Moreover the reaction converting benzyl thioether to sulfonamide **4.19** was likewise low yielding (~30%). Therefore we decided to use the thiol **4.20** which was synthesized in almost quantitative yield starting from 2',3'-isopropylidene adenosine (**4.11**) and thioacetic acid using Mitsunobu reaction condition followed by deprotection of the acetyl group. Overall synthesis of the sulfonamide **4.19** via 5'-nucleoside thiol **4.20** is depicted in Scheme 4-5. Although the yield for the oxidative chlorination (formation of the intermediate sulfonyl chloride) was very low (~32%), we continued with this protocol.



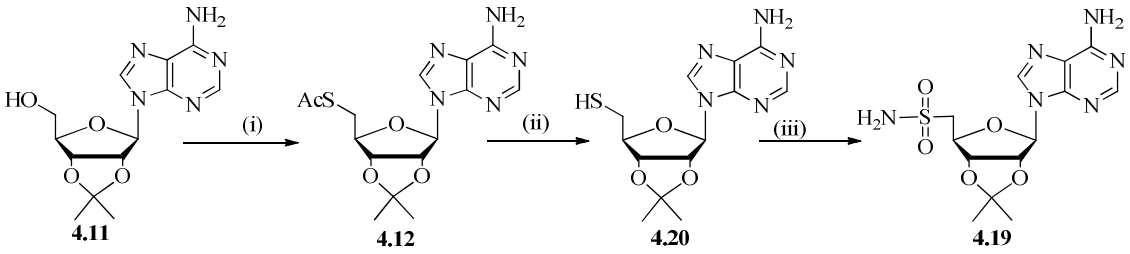
Scheme 4-3: Attempted synthesis of the tetrabutyl ammonium-5'-deoxyadenosine-5'-sulfonate.

Reagents and conditions: (i) Dimethoxy propane, Acetone, PTSA, rt, 16 h; (ii) DEAD, PPh_3,AcSH; (iii) HCOOH, water, rt, 44 h; (iv) Ac_2O, Pyridine, 0° C to rt, 24 h; (v) H_2O_2, TBA acetate, AcOH, 40° C, 12 h (vi) oxone, TBA acetate, glacial acetic acid, rt, 5 h.



Scheme 4-4: Synthesis of the 5'-sulfonamido-5'-deoxyadenosine intermediate **4.19** via the corresponding nucleoside benzylthio ether

Reagents and conditions: (i) $SOCl_2$, acetonitrile, pyridine, 0^o C to 5^o C, 4 h; (ii) BnSH, NaH, DMF, rt, overnight; (iii) PTSA, acetone, DMP, rt, overnight (iv) PPh_3, DEAD, BnSH, THF, 0^o C 1 h; (v) (a) DCDMH, ACN:AcOH:H_2O (40:1.5:1), 0^o C, 2 h, (b) aq. ammonia, 0^o C to rt, overnight.

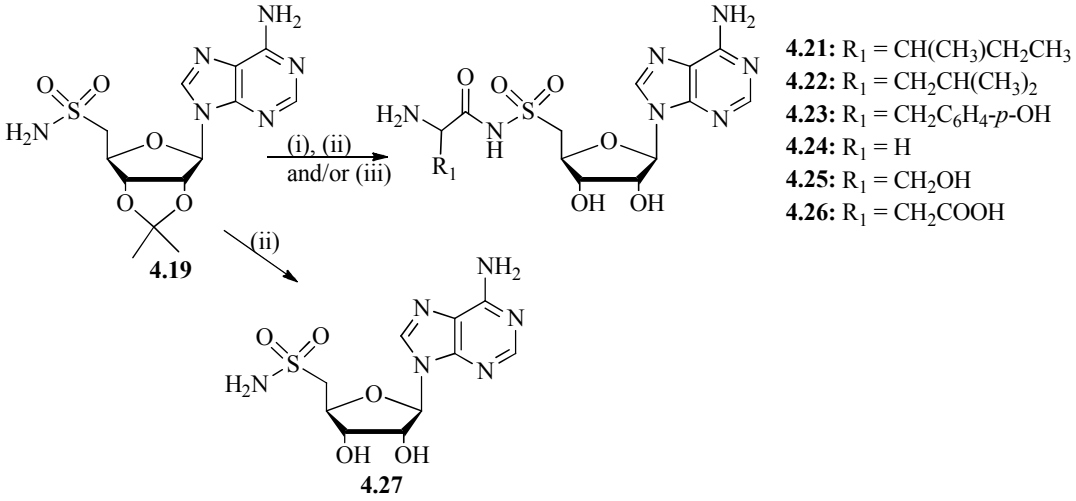


Scheme 4-5: Synthesis of 5'-sulfonamido-5'-deoxyadenosine intermediate via nucleoside thiol.

Reagents and conditions (i) PPh_3, DEAD, thiolacetic acid, THF, 0^o C, 1.5 h; (ii) methanolic ammonia/aq. ammonia (1:1) 0^o C to rt, overnight; (iii) (a) $CH_3CN/AcOH/H_2O$ (40:1.5:1v/v), DCDMH, 1 h; (b) liquid ammonia, 0^o C to rt, 1 h.

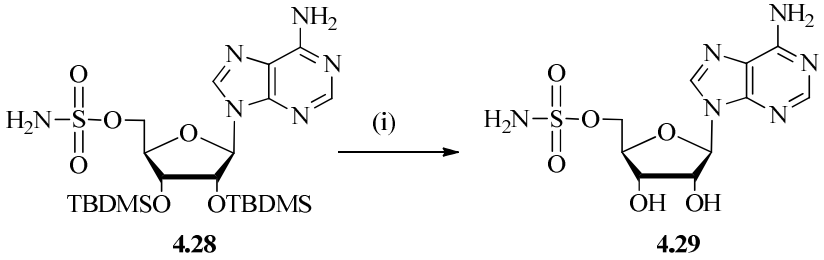
Having the sulfonamide in hand, aa-sulfonamides were synthesized analogously to a literature procedure[82] but using orthogonal protecting groups for the side chain with respect to the α-amino group (Scheme 4-6). The aaSoA **4.21**-**4.26** were obtained by coupling of the respective *N*-hydroxy succinimide ester of appropriately protected amino acids with the sulfonamide **4.19** using DBU as a base in DMF. The Boc, ^{t}Bu and acetonide protecting groups were cleaved by acidolysis followed by hydrogenolysis of the

obtained intermediate in a mixture of methanol-water containing catalytic acetic acid and affording target compounds **4.21**-**4.26**. Similarly, acidolysis of intermediate **4.19** afforded the sulfonamide **4.27**.



Scheme 4-6: Synthesis of the desired aa-sulfonamides.
Reagents and conditions: (i) Boc/Cbz-aa-(tBu/Bn)-OSu, DBU, DMF, 6-8 h; (ii) for isopropylidene, Boc and tBu group deprotection; TFA/water (5/2 v/v), rt, 2.5 h (iii) Pd/C, methanol, cat. acetic acid, H_2 atm. rt, overnight.

Analogously, an intermediate sulfamate **4.29** was synthesized as outlined in Scheme 4-7. As the synthesis of aaSAs has been well described before, it will not be discussed here.[92c, 159]

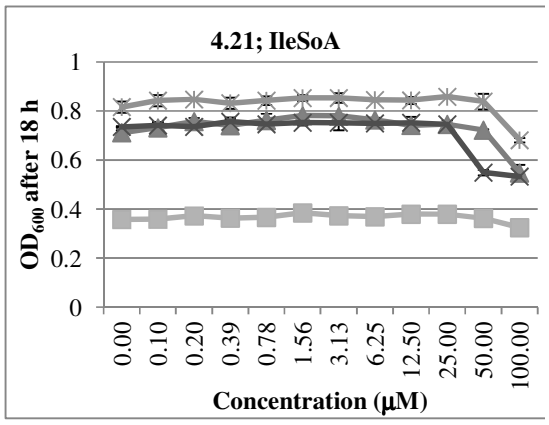


Scheme 4-7: Synthesis of sulfamate (**4.29**).
Reagent and conditions: (i) $Et_3N.3HF$. THF, rt 24 h.

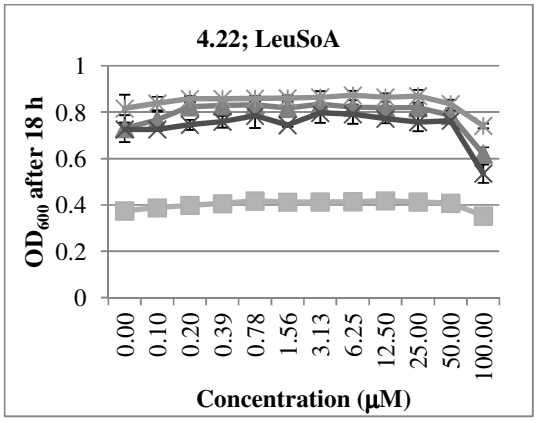
4.4 Antibacterial assay

The antibacterial activities of all newly synthesized aa-sulfonamides against *E. coli* K-12 BW28357, *S. aureus* (ATCC6538), *Sarcina lutea* (ATCC 9341) and *C. albicans* CO11 were determined by measuring the optical density reached by the cell suspension in the wells of microtiter plates in the presence of various concentrations of the respective inhibitors. These four strains were selected to cover the spectrum of activity going from Gram-negative (*E. coli*) to Gram-positive strains (*S. aureus* and *S. lutea*), and to fungi (*C. albicans*). All strains were grown on LB medium. As can be seen from Figure 4-3, only the sulfamate **4.29** showed growth inhibitory activity against all tested strains (panel H) and aspartyl-sulfonamide **4.26** was active only against *E. coli* K-12 (panel F). The likely reason for inactivity could be the lack of cell-penetration of these analogues (similar to aaSA analogues) or loss of affinity of the compound due to deletion of the 5'-oxygen. It has been reported in the literature that ascamycin could not penetrate the cell membrane whereas its dealanyl analogue is able to penetrate the cell membrane and showed broad-spectrum of antibacterial activity. The sulfamate analogue (**4.29**) which closely resembles the dealanyl ascamycin (except chloro substitution at C-2) analogously showed broad spectrum of activity against all tested strains. However, only the AspSoA analogue **4.26** showed selective toxicity against *E. coli* indicating that these analogues apparently can be transported across the cell membrane and resulting to the loss of recognition by the respective aaRS enzymes. The results of broth dilution test are summarized in Figure 4-3 and Table 4-2.

A)



B)



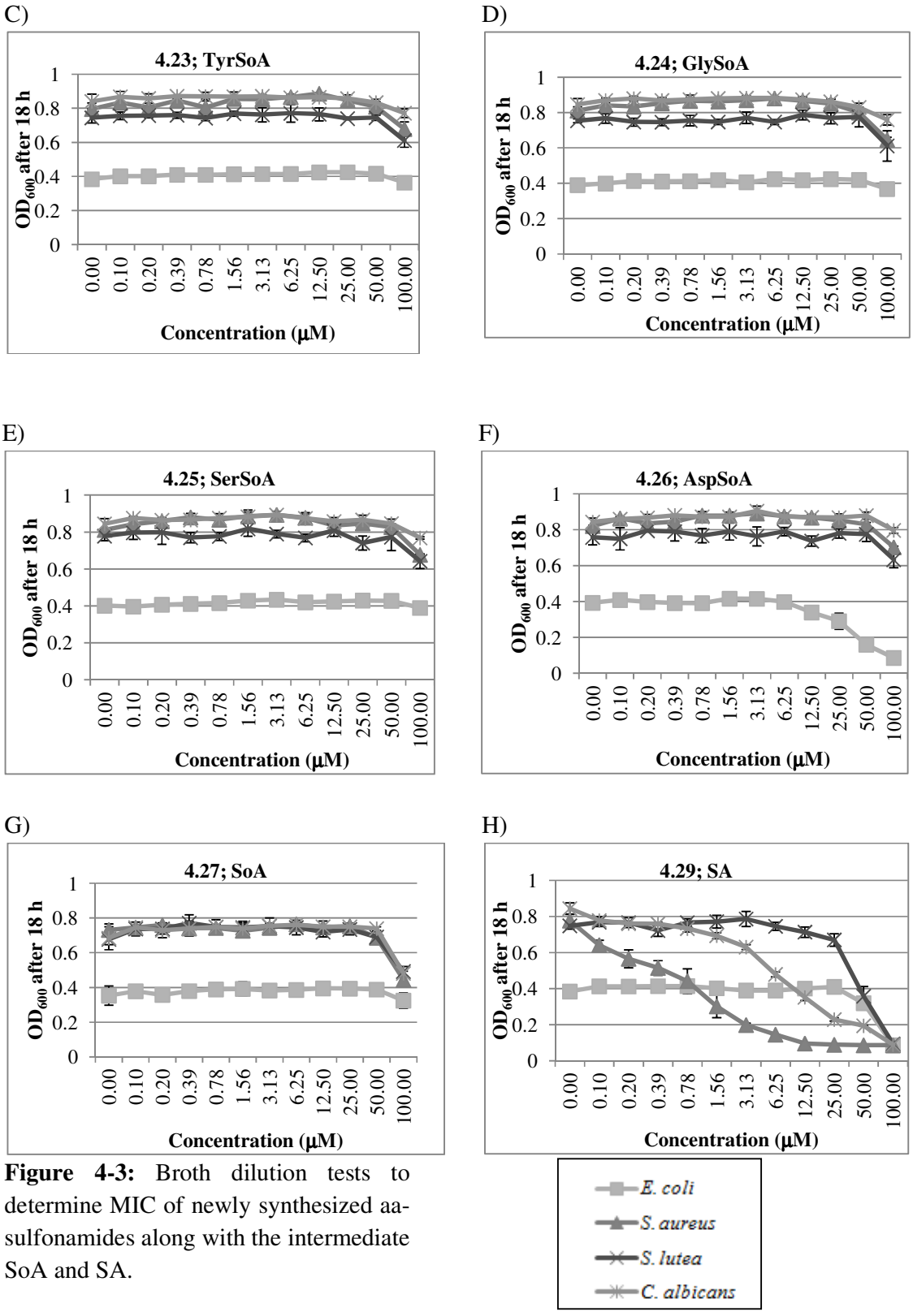


Figure 4-3: Broth dilution tests to determine MIC of newly synthesized aa-sulfonamides along with the intermediate SoA and SA.

Table 4-2: MIC_{50} values of aaSoAs, SoA and SA analogues against different microorganisms

		MIC_{50} (μM)			
SN	Compound*	*E. coli* wt	*S. aureus*	*S. lutea*	*C. albicans*
1.	**4.21**	>100	>100	>100	>100
2.	**4.22**	>100	>100	>100	>100
3.	**4.23**	>100	>100	>100	>100
4.	**4.24**	>100	>100	>100	>100
5.	**4.25**	>100	>100	>100	>100
6.	**4.26**	~ 50 μM	>100	>100	>100
7.	**4.27**	>100	>100	>100	>100
8.	**4.29**	~75 μM	~1 μM	~50 μM	~10 μM

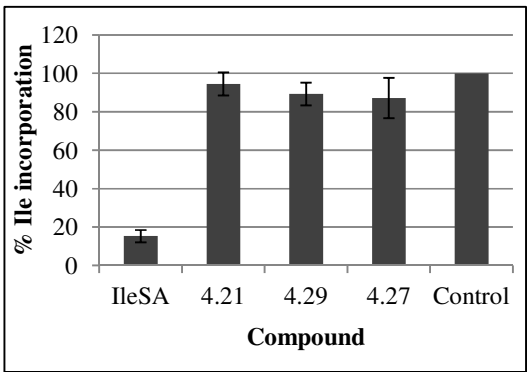
*Maximum inhibitor concentration tested was 100 μM.

To understand the reason(s) for this disappointing lack of antibacterial activity of all newly synthesized aaSoAs and to investigate the probable mode of action of the sulfamate **4.29**, *in vitro* aminoacylation experiments were performed. Hereto, the ability of all compounds to inhibit the corresponding aaRS in *E. coli* wt extract was determined. As shown earlier for aaSA analogues, the intracellular target for the respective aaSA analogue is determined by the amino acid moiety attached to sulfamoyl adenosine. Therefore, aaSoAs were evaluated for their ability to inhibit the incorporation of the corresponding amino acid. The respective aaSA analogues were used as a control to compare the activity of the newly synthesized aaSoAs. Moreover, the plain SoA **4.27** and sulfamate **4.29** were included in the evaluation against all six aaRSs (Figure 4-4).

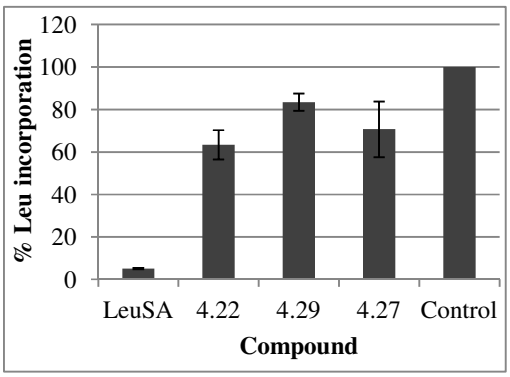
From the *in vitro* experiments, it can be concluded that deletion of the 5'-oxygen of the sulfamate leads to a significant reduction in the inhibitory activity of these analogues as compared to the respective aaSA analogue (with notable exception as found for the AspSoA, **4.26**). Removal of the amino acid part of the aaSoA and aaSA analogues provided SoA **4.27** and sulfamate **4.29** respectively, but these lead to further reduction in inhibitory activity against all tested aaRSs as noted in our test panel. To our surprise, the sulfamate **4.29** which showed antibacterial activity against all tested strains was found to be inactive against all six aaRS under *in vitro* aminoacylation experiment implying that the sulfamate (aaSA but lacking amino acid part) has a different mode of action. As reported earlier, ascamycin is a natural antibiotic which has a narrow spectrum of activity and showed selective toxicity against Xanthomonas species whereas its dealanyl analogue is a broad spectrum antibiotic. Ascamycin and its dealanyl analogue are known to act by

inhibiting polyuridylate directed polyphenylalanine synthesis. We therefore, we tested **4.27** and **4.29** for their ability to inhibit phenylalanine incorporation using PheSA as a control. The results of these in vitro aminoacylation experiments are summerized in Figure 4-4.

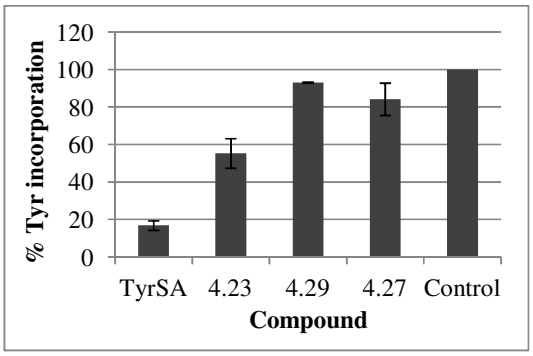
A) IleRS inhibition



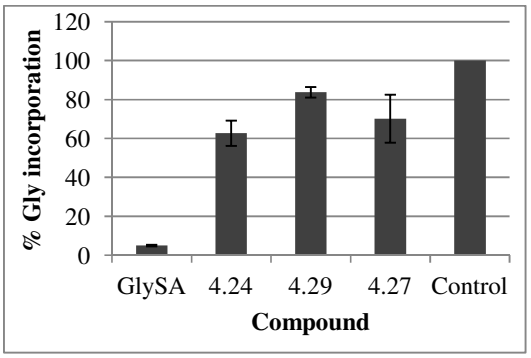
B) LeuRS inhibition



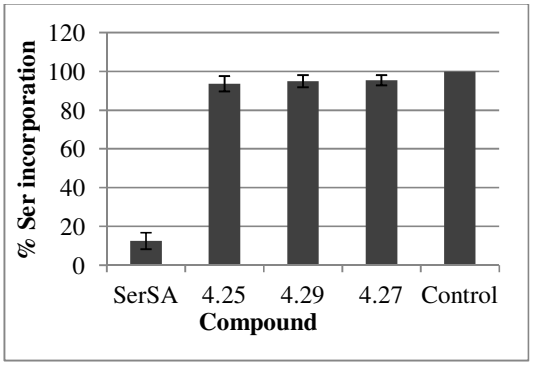
C) TyrRS inhibition



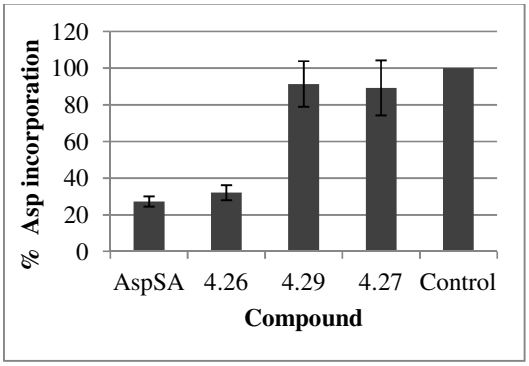
D) GlyRS inhibition



E) SerRS inhibition



F) AspRS inhibition



G) PheRS inhibition

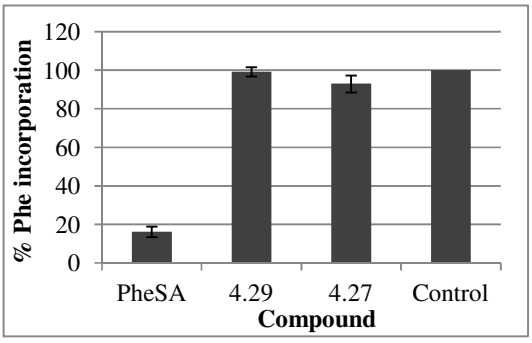


Figure 4-4: *in vitro* aminoacylation experiments using *E. coli* wt S30 cell extracts at 2.5 μM final concentration of respective inhibitor: panel **A)** IleRS, **B)** LeuRS, **C)** GlyRS, **D)** SerRS, **E)** TyrRS **F)** AspRS **G)** PheRS inhibition. The respective aaSA analogues were used as a positive control.

4.5 Discussion

As shown before, synthesis of aaSA analogues is cumbersome due to their chemical instability. Therefore, aaSoAs have been put forward as a more stable alternative to aaSA analogues. It can be seen from broth dilution test results that only the sulfamate analogue **4.29** is active against all tested strains whereas AspSoA **4.26** is active against *E. coli* wt. The likely reason for the lack of inhibitory activity of the other aaSoA could be either lack of cell penetration or lack of affinity for their hypothesized target. However, unlike aaSA analogues, aspartyl-sulfonamide did show some activity against *E. coli* wt at a higher micromolar concentration (~50 μM) indicating that this analogue is able to cross the cell membrane (at least Gram-negative bacteria). Therefore, the loss of activity most likely should be attributed to a loss of binding affinity for the target.

To find out the reason for this lack of antibacterial activity and to study the mode of action of the sulfamate **4.29**, *in vitro* aminoacylation experiments were carried out. These showed that the newly synthesized aaSoA (except **4.26**) lack inhibitory activity against their hypothesized target. Moreover, the compounds **4.29** and **4.27** were inactive against all tested aaRS. Therefore, we assumed that AspSoA **4.26** may act by inhibiting AspRS whereas loss of affinity for the respective aaRS is the most likely reason for inactivity of all other aaSoAs. However, the broad-spectrum activity of the sulfamate **4.29** remained unexplained. It has been reported that ascamycin is a natural antibiotic structurally resembling AlaSA and showing selective toxicity against *Xanthomonas* species (due to the presence of an ascamycin dealanylating enzyme, Xc-aminopeptidases) whereas its dealanyl analogue exhibits broad-spectrum antibacterial activity.[55] These results illustrated that the intact ascamycin could not penetrate the cell-membrane but

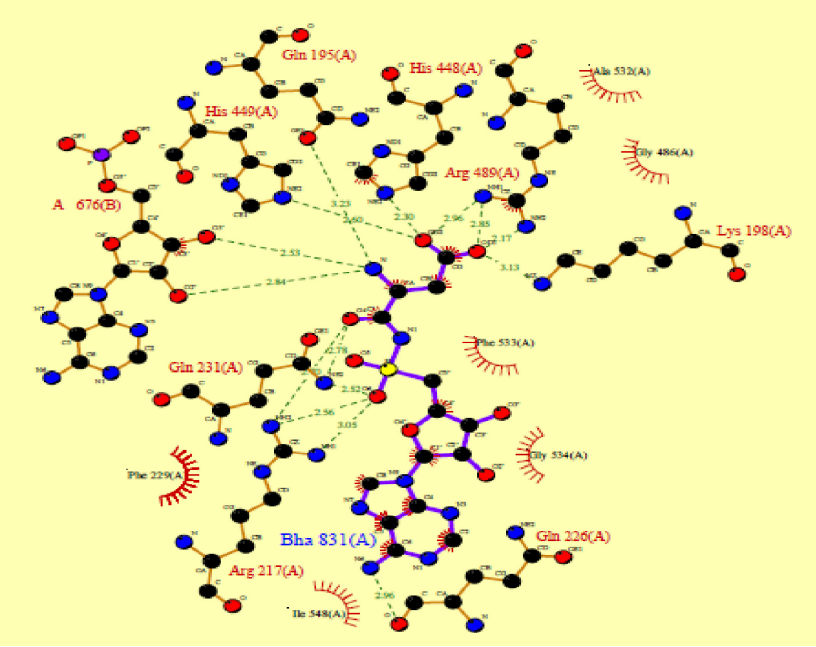
after dealanylation, becomes permeable and displaying antibacterial activity. Both ascamycin and dealanyl ascamycin are known to act by inhibiting polyuridylate directed polyphenylalanine synthesis. Therefore, sulfamate **4.29** and SoA **4.27** were evaluated for their ability to inhibit phenylalanine incorporation under *in vitro* aminoacylation conditions. PheSA was used as a positive control (panel G, Figure 4-4). From these experiments, we concluded that the sulfamate **4.29** although closely resembling to dealanyl-ascamycin exercise its inhibitory effect via a different mode of action than by inhibiting PheRS. Further research is needed to resolve this issue.

There are several reasons which can be proposed for the loss of activity of aaSoAs as compared to their respective aaSA analogues. Most likely reason could be the shortened linker length of aaSoA as compared to aaSA analogues which in turn could lead to the loss of H-bonding with the α-amino group of aaSoAs. We recently established that the α-amino group is an important recognition point at the active site of aaRS when studying *N*-methylation which annihilated the inhibitory activity.[160] Alternatively, deletion of the 5'-oxygen provides aaSoA which possibly fail to mimic the stereoelectronic properties of the sulfamate. It has been reported in the literature that the distance between amino acid and the sugar moiety is crucial for tight binding.[39c] Moreover, the degree of puckering in the ribose ring also plays an important role to achieve the desired accuracy in the aminoacylation reaction.[25] Elimination of the 5'-oxygen leads to a decreased distance between the sugar and the amino acid which may result in steric clashes at the amino acid binding site of aaRS.

Recently, Pope *et al.* developed a binding model for mupirocin bound to IleRS. Based on this model they have designed IleRS inhibitors combining the structural features of mupirocin and IleSA.[39c] They further optimized the length and polarity of the linker for IleRS inhibition. According to their data, the distance between the sugar and the Ile moiety is crucial for tight binding. An increased distance herein resulted in significant reduction in inhibitory activity. Moreover, replacement of the linking oxygen by a methylene or by a nitrogen atom reduced the potency dramatically. They concluded that the stereoelectronic properties of the sulfamate are optimal to mimic the acyl-phosphate intermediate in the aminoacylation reaction.[39c] Furthermore, replacement of the sulfamate oxygen with –NHO- or substituting the sulfamate linkage with an ester or

amide groups yielded analogues with either increased or decreased distance between the sugar and the amino acid, both leading to a significant decrease in potency.[58b, 60] Moreover, replacement of the sulfamate oxygen with nitrogen also lead to a significant loss in activity implying that sulfamide analogues although having the same length, failed to mimic the negative charge density of the acyl phosphate of aa-AMP. Our results are in good agreement with the literature. However, whether it is the overall length or the charge around the sulfonamide linkage which are the main culprit for the affinity loss of these analogues remains to be determined. Therefore, the study of homoadenosine analogues (**4.2b**, Figure 4-1) occupying the same length as that of aaSA analogues is warranted.

To our surprise, only aspartic acid derivative **4.26** displayed some selective toxicity against *E. coli* wt in a whole-cell assay and also showed AspRS inhibition in the *in vitro* aminoacylation experiment. In order to get some insight into the binding mode of the AspSoA in the active site of AspRS, we performed some molecular simulation on *E. coli* AspRS (PDB code 1c0a).[75] It can be seen from the ligplot of the AspSoA (Figure 4-5a) and AspSA (Figure 4-5b), and superimposition of both analogues at the active site (Figure 4-5c) that the binding mode of AspSA and AspSoA are quite analogous. No steric clashes were observed during docking of the AspSoA within the active site of AspRS.



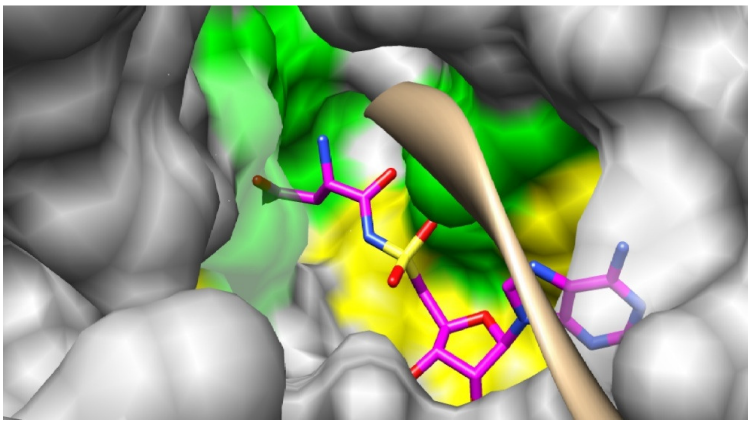
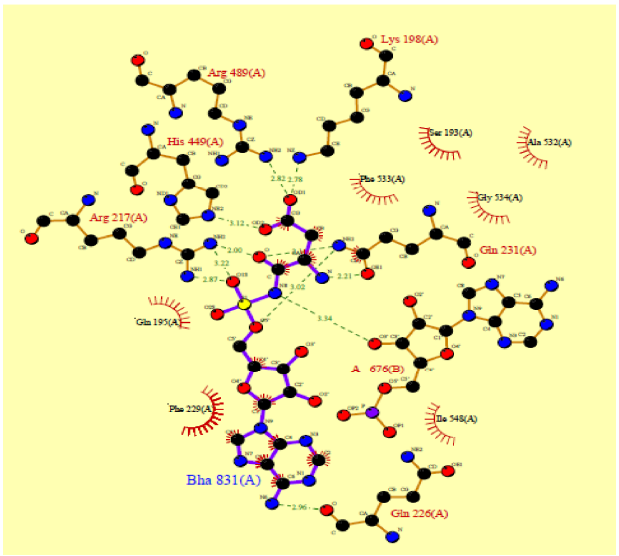


Figure 4-5a: Aspartyl-sulfonamide bound to AspRS from *E. coli* wt (upper panel: ligplot and lower panel: interactive hydrophobic surface).



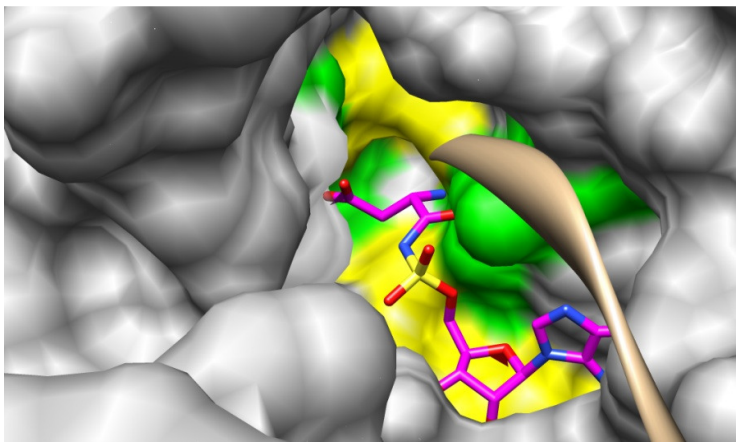


Figure 4-5b: AspSA bound to AspRS from *E. coli* wt (upper panel: ligplot showing interaction and lower panel: interactive hydrophobic surface).

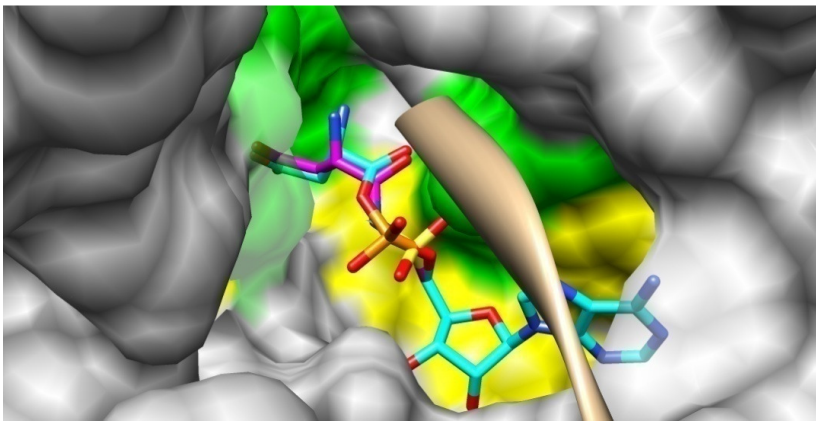


Figure 4-5c: Superimposition of AspSoA and AspSA in the active site of aaRS

4.6 Conclusions

Very few examples of nucleoside sulfonamides have been reported in the literature probably due to difficulties in their synthesis. We also encountered similar difficulties in the synthesis of the SoA intermediate. Among the several attempts undertaken, oxidative chlorination using DCDMH seems a promising and straightforward strategy for the synthesis of nucleoside sulfonamides. The reaction conditions needs to be further optimized to improve the yields of the desired sulfonamides.

Several aaSoA analogues have been synthesized and evaluated for antibacterial activity. Unfortunately, no inhibitory activity was observed for these aaSoAs analogues (except for the AspSoA) either in *in vitro* or in whole-cell assays. From the *in vitro* experiments it can be concluded that the loss of affinity for the target is the most likely reason for inactivity of these analogues. However, whether it is the overall length or the charge around the sulfonamide which is the main culprit for the affinity loss remains unclear. Interestingly, the SA core structure did show broad-spectrum antibacterial activity in a whole-cell assay, but is acting via a different mechanism and not via inhibition of an aaRS enzyme.

4.7 Experimental section

4.7.1 Materials and Methods

Analogous to the procedures described in section 2.5.1

Sodium-5'-deoxyadenosine-5'-sulfonate (4.8)

Adenosine **4.5** (2.14 g, 8.0 mmol) and triphenylphosphine (3.04, 12.0 mmol) were suspended in dry pyridine (20 mL) and stirred for 15 min at room temperature. Hereto, suspension, iodine (3.14 g, 12.0 mmol) was added and reaction mixture was stirred for an additional 2 h at room temperature. Next, the solvent was evaporated and the crude product obtained was used as such without purification. The crude product was dissolved in a mixture of methanol: water (30: 50 mL). To this sodium sulphite (3.0 g, 24 mmol) was added and the mixture was heated to reflux under inert atmosphere for 24 h. Next, the methanol was evaporated under reduced pressure and water layer was washed with ethyl acetate (2x100 mL). The aqueous layer was collected and evaporated to dryness. The obtained residue was suspended in the minimum amount of methanol and filtered through celite and celite was washed with methanol. The filtrate was collected and evaporated to dryness. The yellow residue was subjected to silica gel column chromatography to yield 1.39 g (3.93 mmol, 49%) of the sulfonate derivative as a pale yellow solid.

^{1}H NMR (300 MHz, D_2O) δ 3.34-3.36 (m, 2H, H-5'), 4.36 (t, 1H, *J* = 5.1 Hz, H-3'), 4.42-4.45 (m, 1H, H-4'), 5.99 (d, 1H, *J* = 5.1 Hz, H-1'), 8.12 (s, 1H, H-8), 8.22 (s, 1H, H-2), H-2' merged in solvent signal; ^{13}C NMR (75 MHz, D_2O) δ 53.2 (C-5'), 72.5 (C-3'), 78.8 (C-2'), 79.8 (C-4'), 87.5 (C-1'), 139. 9 (C-8), 148.5 (C-4), 152.5 (C-2), 155.2 (C-6); HRMS for $C_{10}H_{12}N_5Na_2O_6S$ ($[M+Na]^+$) calcd: 376.0299 found 376.0298.

Sodium-2,3-di-*O*-acetyl-N^6,N^6-diacetyl-5'-deoxyadenosine-5'-sulfonate (4.9)

To a solution of **4.8** (525 mg, 1.49 mmol) and DMAP (20 mg, catalytic) in a mixture of dry pyridine and DMF (1:1, 18 mL) at 0^o C was added benzoyl chloride (1.1 mL, 9.0 mmol) over a period of 10 min. The reaction mixture was allowed to warm to room temperature and stirred for 24 h. Next, the solvent was evaporated and the residue was purified by column chromatography to yield 1.01 g (1.31 mmol, 89%) of the title compound as a pale yellow viscous oil.

^{1}H NMR (300 MHz, DMSO-d_6) δ 3.35 (m, 2H, H-5'), 4.78 (m, 1H, H-4'), 6.29 (m, 1H, H-1'), 6.5 (m, 2H, H-2' and H-3'), 7.33-8.02 (28H, Ar), 8.71 (bs, 1H, H-8), 9.01 (bs, 1H, H-2); ^{13}C NMR (75 MHz, DMSO-d_6) δ 53.5 (C-5'), 72.9 (C-3'), 73.9 (C-2'), 80.2 (C-4'), 85.4 (C-1'), 127.3-133.9 (aromatic signals), 146.3 (C-8), 151.0 (C-4), 152.0 (C-2), 152.6 (C-6,), 164.2, 164.7, 172.0 (C=O); HRMS for $C_{38}H_{28}N_5O_{10}S$ ($[M-Na]^-$) calcd: 746.1557 found 746.1558

2',3'-*O*-isopropylidene adenosine (4.11)[161]

PTSA (7.1 g, 37.42 mmol) was added to a suspension of **4.6** (10 g, 37.42 mmol) in acetone (300 mL). Followed by addition of dimethoxy propane (20 mL) the mixture was and allowed to stir at room temperature for overnight. The reaction was monitored by TLC (1:9 MeOH:DCM). After completion of reaction, the reaction was quenched by

adding saturated $NaHCO_3$ (till pH= 8). The acetone was evaporated under reduced pressure and the aqueous layer was washed with ethyl acetate (150 mL x4). The organic layer and washings were collected, dried over anhydrous sodium sulphate, filtered and evaporated to dryness. The product was subjected to column chromatography to yield 9.5 g (30.93 mmol, 83%) of the title compound as a white solid.

^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ 1.33 (s, 3H, C*H*$_3$), 1.55 (s, 3H, C*H*$_3$), 3.48-3.60 (m, 2H, H-5'a and H-5'b), 4.92-5.0 (m, 1H, H-4'), 5.24 (t, 1H, *J* = 5.4 Hz, H-3'), 5.32-5.38 (m, 1H, H-2'), 6.13 (d, 1H, *J* = 3.0 Hz, H-1'), 7.35 (bs, 1H, 6-N*H*$_2$), 8.16 (s, 1H, H-8), 8.35 (s, 1H, H-2); ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ 25.2 (*C*H$_3$), 27.1 (*C*H$_3$), 54.9 (C-5'), 81.4 (C-3'), 83.2 (C-2'), 86.4 (C-4'), 89.6 (C-1'), 113.1 (*C*(CH$_3$)$_3$), 119.1 (C-5), 139.7 (C-8), 148.8 (C-4), 152.6 (C-2), 156.2 (C-6); HRMS for $C_{13}H_{18}N_5O_4$ ([M+H]$^+$) calcd: 308.1353 found 308.1352.

5'-deoxy-2',3'-*O*-isopropylidene-5'-thioacetyl-adenosine (4.12)[155]

To an ice-cold solution of triphenylphosphine (3.76 g, 14.32 mmol) in dry THF (20 mL), diethyl azodicarboxylate (2.2 mL, 14.32 mmol) was added over 5 min. After stirring for 30 min, **4.11** (2.0 g, 6.51 mmol) was added, and stirring was continued for 10 min. To the resulting yellow suspension a solution of thioacetic acid (1.0 mL, 14.32 mmol) in dry THF (5 mL) was added drop wise and stirring was continued for another 1.5 h at 0°C. During this time the yellow suspension cleared, and an orange solution was obtained. At the end of the reaction the solvent was removed under reduced pressure, and the resulting yellowish residue was purified by flash chromatography on silica gel. The column was eluted with $CHCl_3$:THF (4:1 v/v) followed by a gradient of 2-10% methanol in $CHCl_3$. The product containing fractions were evaporated to afford 2.35 g (6.43 mmol, 99%) of the title compound as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.39 (s, 3H, C*H*$_3$), 1.60 (s, 3H, C*H*$_3$), 2.34 (s, 3H, COC*H*$_3$), 3.19 (dd, 1H, *J* = 6.9 Hz and 13.5 Hz, H-5'a), 3.30 (dd, 1H, *J* = 6.9 Hz and 13.5 Hz, H-5'b), 4.35 (dt, 1H, *J* = 3.3 Hz, H-4'), 4.98 (dd, 1H, *J* = 3.0 Hz, H-3'), 5.53 (dd, 1H, *J* = 6.6 Hz and 2.1 Hz, H-2'), 5.66 (bs, 2H, 6-N*H*$_2$), 6.06 (d, 1H, *J* = 2.1 Hz, H-1'), 7.90 (s, 1H, H-8), 8.37 (s, 1H, H-2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.5 (*C*H$_3$), 26.2 (*C*H$_3$), 29.7 (CO*C*H$_3$), 30.4 (C-5'), 82.9 (C-3'), 83.4 (C-2'), 85.3 (C-4'), 90.1 (C-1'), 113.7 (*C*(CH$_3$)$_3$), 139.2 (C-8), 152.4 (C-2), 154.7 (C-6), 193.7 (CO), C-4 and C-5 not detected; HRMS for $C_{15}H_{20}N_5O_4S$ ([M+H]$^+$) calcd: 366.1230 found 366.1227.

5'-deoxy-2',3'-*O*-isopropylidene-5'-thioacetyl-adenosine (4.13)

A solution of **4.12** (490 mg, 1.39 mmol) in a mixture of formic acid and water (25 mL, 1:1 v/v) was stirred at room temperature for 44 h. The progress of the reaction was monitored by TLC (10% MeOH:DCM). Next, the solvent was evaporated under reduced pressure, and traces of formic acid were removed by coevaporating five times with absol.

ethanol. The obtained white powder was purified by silica gel chromatography to afford 329 mg (1.01 mmol, 75%) as a white solid.

^{1}H NMR (300 MHz, DMSO-d_6) δ 2.33 (s, 3H, SCOCH_3), 3.14-3.18 (m, 2H, H-5'), 3.90-3.94 (m, 1H, H-4'), 4.09-4.10 (m, 1H, H-3'), 4.79 (q, 1H, J = 5.0 Hz, H-2'), 5.37 (d, 1H, J = 4.5 Hz, 3'-OH), 5.51 (d, 1H, J = 5.5 Hz, 2'-OH), 5.87 (d, 1H, J = 6.0 Hz, H-1'), 7.29 (s, 2H, NH_2), 8.15 (s, 1H, H-8), 8.34 (s, 1H, H-2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 30.5 (COCH$_3$), 31.3 (C-5'), 72.6, 72.7 (C-2' and C-3'), 82.9 (C-4'), 87.6 (C-1'), 119.3 (C-5), 140.0 (C-8), 149.5 (C-4), 152.7 (C-2), 156.2 (C-6), 194.8 (COCH$_3$); HRMS for $C_{12}H_{16}N_5O_4S$ ([M+H]$^+$) calcd: 326.0917 found 326.0921.

5'-deoxy-2',3'-di-*O*-acetyl-N^6,N^6-diacetyl-5'-thioacetyl-adenosine (4.14)

Acetic anhydride (556 μL, 5.88 mml) was added to an ice-cold solution of **4.13** (320 mg, 0.98 mmol) and DMAP (10 mg, catalytic) in dry pyridine (5 mL). The reaction mixture was allowed to warm to room temperature and stirred for 24 h. Next, the reaction mixture was cooled to 0^o C and quenched by addition of ethanol (0.5 mL). The solvent was evaporated to dryness and the residue was partitioned between ethyl acetate and saturated sodium bicarbonate. The organic layer was further washed with 1N HCl. The organic layer was collected, dried over anhydrous sodium sulphate, filtered and evaporated to dryness. The residue obtained was purified by silica gel chromatography. After purification, 120 mg (0.27 mmol, 27 %) of the N^6-acetyl derivative and 200 mg (0.41 mmol, 41%) of N^6,N^6-bisacetyl derivative (desired product) were obtained as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 2.10 (s, 3H, COCH_3), 2.16 (s, 3H, COCH_3), 2.38 (s, 9H, 3xCOCH_3), 3.35-3.52 (m, 2H, H-5'), 4.43 (q, 1H, J = 5.4 Hz, H-4'), 5.57 (t, 1H, J = 5.4 Hz, H-3'), 6.01 (t, 1H, J = 5.1 Hz, H-2'), 6.20 (d, 1H, J = 5.1 Hz, H-1'), 8.31 (s, 1H, H-8), 8.99 (s, 1H, H-2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 19.5 (COCH_3), 19.6 (COCH_3), 25.4 (2xCOCH_3), 29.6 (COCH_3), 29.9 (C-5'), 71.1 (C-3'), 72.1 (C-2'), 80.2 (C-4'), 86.0 (C-1'), 130.0 (C-5), 143.8 (C-8), 150.2 (C-4), 152.0 (C-2), 152.2 (C-6), 168.4, 168.6, 170.9, 193.5 (COCH3); HRMS for $C_{20}H_{24}N_5O_8S$ ([M+H]$^+$) calcd: 494.1340 found 494.1340.

5'-chloro-5'-deoxyadenosine (4.16)

Adenosine **4.6** (500 mg, 1.87 mmol) was suspended in a mixture of pyridine (0.5 mL) and acetonitrile (6.5 mL). This mixture was cooled to 0°C and thionylchloride (0.7 mL, 9.35 mmol, 5 eq.) was added over a period of 5 min. This mixture was stirred for 4 h at 5°C, after which it was allowed to warm to room temperature and was stirred overnight during which a white precipitate formed. The precipitate was filtered and dried. The resulting powder was suspended in a 5:1 mixture of methanol and water (12 mL) and a concentrated solution of ammonia in water (0.94 mL) was added. The solvent was evaporated under reduced pressure, yielding the title compound 196 mg (0.69 mmol, 37%) as a white solid.

^{1}H NMR (300 MHz, DMSO-d_6) δ 3.83-3.97 (m, 2H, H-5'), 4.06-4.11 (m, 1H, H-4'), 4.19-4.24 (m, 1H, H-3'), 4.72-4.78 (m, 1H, H-2'), 5.43-5.59 (d, 2H, 2'-OH and 3'-OH), 5.92 (d, 1H, J = 5.4 Hz, H-1'), 7.30 (bs, 2H, NH_2), 8.15 (bs, 1H, H-8), 8.33 (bs, 1H, H-2); ^{13}C NMR (75MHz, DMSO-d_6) δ 45.0 (C-5'), 71.4 (C-3'), 72.8 (C-2'), 83.8 (C-4'), 87.6 (C-1'), 119.3 (C-5), 139.9 (C-8), 149.6 (C-4), 152.9 (C-2), 156.2 (C-6); HRMS for $C_{10}H_{13}ClN_5O_3$ ([M+H]$^+$), calcd: 286.0701 found 286.0701.

2',3'-*O*-isopropylidene-5'-thiobenzyl-5'-deoxyadenosine (4.18)

To an ice-cold solution of triphenylphosphine (940 mg, 3.6 mmol) in dry THF (5 mL), diethyl azodicarboxylate (0.57 mL, 3.6 mmol) was added over 5 min. After stirring for 30 min, **4.11** (500 mg, 1.63 mmol) was added, and stirring was continued for 10 min. To the resulting yellow suspension a solution of benzylmercaptan (423 μL, 3.6 mmol) in dry THF (2 mL) was added drop wise and stirring was continued for another 1 h at 0°C. During this time the yellow suspension cleared, and an orange solution was obtained. At the end of the reaction the solvent was removed under reduced pressure, and the resulting yellowish residue was purified by flash chromatography on silica gel to yield 273 mg (0.66 mmol, 41%) of the title compound as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.39 (bs, 3H, CH_3), 1.61 (bs, 3H, CH_3), 2.63-2.81 (m, 2H, H-5'), 3.71 (bs, 2H, CH_2-Ph), 4.33-4.38 (m, 1H, H-3'), 4.99 (dd, 1H, *J* = 3.3 Hz and *J* = 6.3 Hz, H-4'), 5.46 (dd, 1H, *J* = 2.1 Hz and *J* = 6.3 Hz, H-2'), 5.54 (bs, 2H, NH_2), 6.06 (d, 1H, *J* = 2.4 Hz, H-1'), 7.19-7.26 (m, 5H, Ph), 7.91 (bs, 1H, H-8), 8.31 (bs, 1H, H-2); ^{13}C NMR (75 MHz, $CDCl_3$) d 25.6 (*C*H_3), 27.4 (CH3), 33.5 (C-5'), 36.7 (*C*H_2Ph), 84.1 (C-3'), 84.2 (C-2'), 86.9 (C-4'), 91.0 (C-1'), 114.8 (quaternary C, acetonide), 127.4, 128.8, 129.1 (C-5 and aromatic), 140.3 (C-8), 153.4 (C-2); HRMS for $C_{20}H_{24}N_5O_3S$ ([M+H]$^+$), calcd: 414.1594, found: 414.1593.

5'-deoxy-2',3'-*O*-isopropylidene-5'-mercapto-adenosine (4.20)[155]

Compound **4.12** (8.4 g, 22.99 mmol) was dissolved in a mixture of methanolic ammonia: aqueous ammonia (1:1, 100 mL) and was stirred at 0^o C for 1h. After 1 h, the ice-bath was removed and the reaction mixture was stirred at room temperature for 24 h while monitoring the reaction for completion. The solvent was evaporated under reduced pressure and the product was purified by column chromatography to yield 6.9 g (21.34 mmol, 93 %) of the title compound as a foam.

^{1}H NMR (300 MHz, DMSO-d_6) δ 1.32 (s, 3H, CH_3), 1.52 (s, 3H, CH_3), 2.96 (dd, 1H, *J* = 7.0 Hz and 13.5 Hz, H-5'a), 3.04 (dd, 1H, *J* = 7.5 Hz and 14.0 Hz, H-5'b), 4.33 (dt, 1H, *J* = 2.5 Hz and 4.5 Hz, H-4'), 5.01 (dd, 1H, *J* = 2.5 Hz and 6.0 Hz, H-3'), 5.50 (dd, 1H, *J* = 2.0 Hz and 6.0 Hz, H-2'), 5.75 (bs, 1H, SH), 6.17 (d, 1H, *J* = 2.0 Hz, H-1'), 7.34 (bs, 2H, 6-NH_2), 8.17 (s, 1H, H-8), 8.30 (s, 1H, H-2); ^{13}C NMR (75 MHz, DMSO-d_6) δ 25.2 (*C*H_3), 26.9 (*C*H_3), 55.0 (C-5'), 83.26 (C-3'), 83.32 (C-2'), 84.8 (C-4'), 89.4 (C-1'), 113.4

(*C*(CH_3)$_3$), 119.3 (C-5), 140.2 (C-8), 148.8 (C-4), 152.8 (C-2), 156.2 (C-6); HRMS for $C_{26}H_{33}N_{10}O_6S_2$(disulfide) ([M+H]$^+$) calcd: 645.2020 found 645.2023.

5'-(sulfonamido)-2',3'-*O*-isopropylidene-5'-deoxyadenosine (4.19)

To an ice-cold solution of **4.20** (6.9 g, 21.34 mmol) in a mixture of CH_3CN-HOAc-H_2O (104.0 mL-4.0 mL– 2.7 mL) was added 1,3-dichloro-5,5-dimethylhydantoin (6.31 g, 32.01 mmol) in two portions over 5 min. The cooling bath was removed and the reaction mixture was allowed to warm to 20^o C and was further stirred for 1 h. Next, the reaction mixture was added drop wise to an ice-cold aqueous ammonia solution over a period of 30 min and was stirred at 0^o C for an additional hour. The ice-bath was removed and the reaction mixture was stirred at room temperature overnight. The next day, the solvent was evaporated to dryness and the residue was subjected to column chromatography to afford 2.53 g (6.84 mmol, 32%) of the title compound as a pale yellow solid.

^{1}H NMR (500 MHz, DMSO-d_6) δ 1.33 (s, 3H, CH_3), 1.54 (s, 3H, CH_3), 3.21 (dd, 1H, *J* = 6.5 Hz and 14.0 Hz, H-5'a), 3.58 (dd, 1H, *J* = 6.5 Hz and 14.0 Hz, H-5'b), 4.58 (dt, 1H, *J* = 2.5 Hz and 6.0 Hz, H-4'), 5.17 (dd, 1H, *J* = 3.0 Hz and 6.5 Hz, H-3'), 5.50 (dd, 1H, *J* = 2.0 Hz and 6.0 Hz, H-2'), 6.21 (d, 1H, *J* = 2.0 Hz, H-1'), 6.92 (s, 2H, SO_2NH_2), 7.36 (s, 2H, 6-NH_2), 8.18 (s, 1H, H-8), 8.33 (s, 1H, H-2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 25.2 (*C*H_3), 26.9 (*C*H_3), 57.4 (C-5'), 81.9 (C-3'), 83.3 (C-2'), 84.1 (C-4'), 89.6 (C-1'), 113.2 (*C*(CH_3)$_3$), 119.3 (C-5), 140.3 (C-8), 148.7 (C-4), 152.8 (C-2), 156.2 (C-6); HRMS for $C_{13}H_{17}N_6O_5S$ ([M-H]$^-$) calcd: 369.0987 found 369.0985.

5'-(*N*-L-isoleucyl-sulfonamido)-5'-deoxyadenosine (4.21)

To a solution of sulfonamide **4.19** (100 mg, 0.27 mmol) in dry DMF (2 mL) were added DBU (102 μL, 0.68 mmol) and Boc-Ile-OSu (177 mg, 0.54 mmol). The reaction mixture was stirred at room temperature for 8 h during which the reaction was monitored by TLC. Next, the solvent was evaporated and the product was purified by silica gel column chromatography (0-60% MeOH:DCM). The fractions containing desired product were evaporated yielding the coupled intermediate which was dissolved in a mixture of TFA/water (5:2 v/v, 3.5 mL) at 0^o C and the mixture was stirred at room temperature for 2 h. The volatiles were evaporated under reduced pressure followed by co-evaporation with toluene (2x) and ethanol (2x). The yellow residue obtained was purified by column chromatography and finally with RP HPLC using PLRP-S column to afford 24 mg (0.06 mmol, 20%) of the title compound **4.21** as a white solid.

^{1}H NMR (500 MHz, D_2O) δ 0.75 (t, 3H, *J* = 7.5 Hz, Ile-δ-CH_3), 0.82 (d, 3H, *J* = 7.0 Hz, Ile-γ-CH_3), 1.01-1.11 (m, 1H, Ile-γ-CH_2 Ha), 1.32-1.41 (m, 1H, Ile-γ-CH_2 Hb), 1.79-1.88 (m, 1H, Ile-β-C*H*), 3.57 (d, 1H, *J* = 4.5 Hz, Ile-α-C*H*), 3.73 (dd, 1H, *J* = 3.0 Hz and 15.0 Hz, H-5'a), 3.84-3.91 (m, 1H, H-5'b), 4.44 (t, 1H, *J* = 5.0 Hz, H-4'), 4.49-4.53 (m, 1H, H-3'), 6.07 (d, 1H, *J* = 4.5 Hz, H-1'), 8.23 (s, 1H, H-8), 8.30 (s, 1H, H-2); ^{13}C NMR (125 MHz, D_2O) δ 10.8 (Ile-δ-*C*H_3), 14.3 (Ile-γ-*C*H_3), 24.1 (Ile-γ-*C*H_2), 36.3 (Ile-β-*C*H), 54.6

(Ile-α-*C*H), 60.2 (C-5'), 72.9 (C-3'), 73.0 (C-2'), 79.3 (C-4'), 88.2 (C-1'), 118.9 (C-5), 140.4 (C-8), 148.9 (C-4), 152.9 (C-2), 156.6 (C-6), 175.3 (C=O); HRMS for $C_{16}H_{24}N_7O_6S$ ([M-H]$^-$) calcd: 442.1514 found 442.1518.

5'-(*N*-L-leucyl-sulfonamido)-5'-deoxyadenosine (4.22)

This compound was synthesized analogously to **4.21**. Yield: 48%

^{1}H NMR (600 MHz, D_2O) δ 0.61 (d, 3H, *J* = 6.6 Hz, Ile-δ-CH_3), 0.75 (d, 3H, *J* = 6.6 Hz, Ile-δ-CH_3), 1.03-1.10 (m, 1H, Ile-γ-C*H*), 1.36-1.42 (m, 1H, Ile-β-CH_2 Ha), 1.46-1.54 (m, 1H, Ile-β-CH_2 Hb), 3.51 (dd, 1H, *J* = 4.2 Hz and 9.0 Hz, Ile-α-C*H*), 3.65 (dd, 1H, *J* = 2.4 Hz and 15.0 Hz, H-5'a), 3.94 (dd, 1H, *J* = 9.6 Hz and 15.0 Hz, H-5'b), 4.40 (t, 1H, *J* = 4.8 Hz, H-4'), 4.48-4.50 (m, 1H, H-3'), 4.74 (t, 1H, *J* = 4.8 Hz, H-2'), 6.08 (d, 1H, *J* = 4.8 Hz, H-1'), 8.24 (s, 1H, H-8), 8.29 (s, 1H, H-2); ^{13}C NMR (150 MHz, D_2O) δ 20.0 (Ile-δ-*C*H_3), 21.5 (Ile-δ-*C*H_3), 23.4 (Ile-γ-*C*H), 39.9 (Ile-β-*C*H_2), 53.7 (C-5'), 53.9 (Ile-α-*C*H), 72.6 (C-3'), 72.7 (C-2'), 78.9 (C-4'), 87.6 (C-1'), 118.4 (C-5), 139.9 (C-8), 148.4 (C-4), 152.6 (C-2), 155.3 (C-6), 177.4 (C=O); HRMS for $C_{16}H_{24}N_7O_6S$ ([M-H]$^-$) calcd: 442.1514 found 442.1510.

5'-(*N*-L-tyrosyl-sulfonamido)-5'-deoxyadenosine (4.23)

To a solution of **4.19** (200 mg, 0.54 mmol) in dry DMF (3 mL) were added DBU (164 µL, 1.08 mmol) and Boc-Tyr(Bzl)-OSu (379 mg, 0.81 mmol). The reaction mixture was stirred at room temperature for 6 h during which the reaction was monitored by TLC. Next, the solvent was evaporated and the product was purified by silica gel column chromatography using MeOH:DCM as eluents. Fractions containing the desired product were evaporated to afford the intermediate which was dissolved in a mixture of TFA/water (5:2 v/v, 3.5 mL) at 0^o C and was stirred at room temperature for 2 h. The volatiles were evaporated under reduced pressure followed by coevaporation with toluene (3x) and ethanol (3x). The yellow residue obtained was purified by column chromatography. The fractions containing the desired product were evaporated to yield an intermediate which was dissolved in a mixture of methanol-water (4:1 v/v, 5 mL) containing glacial acetic acid (0.5 mL). To this solution Pd/C (10%w/w, 70 mg) was added and the mixture was stirred under H_2 atmosphere at room temperature for overnight. Next, the catalyst was filtered off and washed with a mixture of methanol: water (1:1 v/v, 10 mL). The solvent was evaporated under reduced pressure followed by coevaporation with toluene (3x) and ethanol (3x) to remove traces of acetic acid and water. The crude product was purified by column chromatography and finally by RP-HPLC to yield 81 mg (0.17 mmol, 31%) of the title compound **4.23** as a white solid.

^{1}H NMR (500 MHz, D_2O) δ 1.88 (dd, 1H, *J* = 10.0 Hz and 14.5 Hz, Tyr-β-CH_2 Ha), 2.67 (dd, 1H, *J* = 3.5 Hz, 14.5 Hz, Tyr-β-CH_2 Hb), 3.57 (dd, 1H, *J* = 2.0 Hz and 15.0 Hz, Tyr-α-C*H*), 3.65 (dd, 1H, *J* = 4.0 Hz and 10.0 Hz, H-5'a), 3.80-3.90 (m, 1H, H-5'b), 4.35 (t,

1H, *J* = 6.0 Hz, H-4'), 4.40-4.45 (m, 1H, H-3'), 5.99 (d, 1H, *J* = 4.0 Hz, H-1'), 7.95 (s, 1H, H-8), 8.29 (s, 1H, H-2); ^{13}C NMR (150 MHz, D_2O) δ 35.5 (Tyr-β-*C*H_2), 53.4 (Tyr-α-*C*H), 57.0 (C-5'), 72.1 (C-3'), 72.5 (C-2'), 78.4 (C-4'), 87.1 (C-1'), 115.4 (Tyr-*ortho*-C), 118.2 (C-5), 125.7 (Tyr-*ipso*-C), 130.0 (Tyr-*meta*-C), 139.6 (C-8), 148.4 (C-4), 152.5 (C-2), 154.5 (Tyr-*para*-C), 155.0 (C-6), 175.3 (C=O); HRMS for $C_{19}H_{23}N_7O_7S$ ($[M-H]^-$) calcd: 492.1307 found 492.1310.

5'-(*N*-L-glycyl-sulfonamido)-5'-deoxyadenosine (4.24)

This compound was synthesized analogously to **4.21**. Yield: 16%

^{1}H NMR (300 MHz, D_2O) δ 3.59 (s, 2H, Gly-α-C*H*$_2$), 3.76-3.82 (m, 2H, H-5'a and H-5'b), 4.42 (t, 1H, *J* = 5.1 Hz, H-4'), 4.51 (q, 1H, *J* = 5.4 Hz, H-3'), 6.06 (d, 1H, *J* = 4.8 Hz, H-1'), 8.18 (s, 1H, H-8), 8.26 (s, 1H, H-2); ^{13}C NMR (125 MHz, D_2O) δ 42.5 (Gly-α-*C*H_2), 54.0 (C-5'), 72.55 (C-3'), 72.63 (C-2'), 78.8 (C-4'), 87.9 (C-1'), 118.4 (C-5), 139.8 (C-8), 148.3 (C-4), 152.4 (C-2), 155.1 (C-6), 172.7 (C=O); HRMS for $C_{12}H_{16}N_7O_6S$ ($[M-H]^-$) calcd: 386.0888 found 386.0894.

5'-(*N*-L-seryl-sulfonamido)-5'-deoxyadenosine (4.25)

This compound was synthesized similar to **4.23**. Yield: 9%

^{1}H NMR (500 MHz, D_2O) δ 3.58-3.64 (m, 2H, H-5'a and H-5'b), 3.69-3.84 (m, 4H, Ser-α-C*H*, Ser-β-C*H*$_2$ and H-4'), 4.41 (q, 1H, *J* = 5.4 Hz, H-3'), 4.46-4.52 (m, 1H, H-2'), 6.06 (d, 1H, *J* = 2.4 Hz, H-1'), 8.22 (s, 1H, H-8), 8.28 (s, 1H, H-2); ^{13}C NMR (150 MHz, D_2O) δ 54.0 (Ser-α-*C*H), 57.3 (C-5'), 61.3 (Ser-β-*C*H_2), 72.7 (C-2' and C-3'), 79.0 (C-4'), 87.9 (C-1'), 118.7 (C-5), 140.1 (C-8), 148.7 (C-4), 152.7 (C-2), 155.4 (C-6), 175.8 (*C*=O); HRMS for $C_{13}H_{18}N_7O_7S$ ($[M-H]^-$) calcd: 416.0994 found 416.0998.

5'-(*N*-L-aspartyl-sulfonamido)-5'-deoxyadenosine (4.26)

This compound was synthesized similar to **4.23**. Yield: 9%

^{1}H NMR (600 MHz, D_2O) δ 2.53 (dd, 1H, *J* = 9.0 Hz and 17.4 Hz, Asp-β-C*H*$_2$ Ha), 2.77 (dd, 1H, *J* = 3.6 Hz and 17.4 Hz, Asp-β-C*H*$_2$ Hb), 3.72-3.82 (m, 2H, H-5'a and H-5'b), 3.89 (dd, 1H, *J* = 3.6 Hz and 8,4 Hz, Asp-α-C*H*), 4.43 (t, 1H, *J* = 5.4 Hz, H-3'), 4.53 (quin, 1H, *J* = 4.2 Hz, H-4'), 6.10 (d, 1H, *J* = 4.8 Hz, H-1'), 8.27 (s, 1H, H-8), 8.33 (s, 1H, H-2); ^{13}C NMR (150 MHz, D_2O) δ 35.9 (Asp-β-*C*H_2), 52.6 (Asp-α-*C*H), 53.7 (C-5'), 72.55 (C-3'), 72.58 (C-2'), 79.0 (C-4'), 87.9 (C-1'), 118.6 (C-5), 140.3 (C-8), 148.3 (C-4), 151.3 (C-2), 154.4 (C-6), 174.7 (C=O), 176.3 (β-COOH); HRMS for $C_{14}H_{18}N_7O_8S$ ($[M-H]^-$) calcd: 444.0943 found 444.0940.

5'-(sulfonamido)-5'-deoxyadenosine (4.27)

Compound **4.19** (70 mg, 0.19 mmol) was dissolved in a mixture of TFA and water (5:2 v/v, 1 mL) at 0^{o} C and stirred at room temperature for 2.5 h. Next, the reaction mixture was co-evaporated with toluene (3 times) and further co-evaporated with ethanol (3 times). The residue was dissolved in ethanol, neutralized with TEA (0.5 mL) and co-evaporated with toluene (2 times). The yellow residue obtained was purified by column chromatography and finally with RP HPLC using a PLRP-S column to yield 24.5 mg (0.07 mmol, 40%) of the title compound as a white solid.

^{1}H NMR (300 MHz, D_2O) δ 3.75-3.91 (m, 2H, H-5'a and H-5'b), 4.46 (t, 1H, *J* = 5.4 Hz, H-4'), 4.53-4.61 (m, 1H, H-3'), 6.07 (d, 1H, *J* = 4.5 Hz, H-1'), 8.17 (s, 1H, H-8), 8.25 (s, 1H, H-2), H-2' merged in D_2O signal; ^{13}C NMR (75 MHz, D_2O) δ 56.4 (C-5'), 72.3 (C-3'), 72.4 (C-2'), 78.5 (C-4'), 80.1 (C-1'), 139.9 (C-8), 148.3 (C-4), 152.4 (C-2), 155.1 (C-6), C-5 not detected; HRMS for $C_{10}H_{15}N_6O_5S$ ($[M+H]^+$) calcd: 331.0819 found 331.0822.

5'-*O*-sulfamoyl-adenosine (4.29)

To a solution of **4.28** (500 mg, 0.87 mmol) in dry THF was added triethylamine trihydrofluoride (1 mL) and the solution was stirred at room temperature for 24 h. Next, the solvent was evaporated and the residue was purified by column chromatography to yield 678 mg of a pale yellow foam. The residue was further purified by HPLC using a PLRP-S column to yield 201 mg (0.58 mmol, 67%) of the title compound as a white solid.

^{1}H NMR (600 MHz, CD_3OD) δ 4.30-4.33 (m, 1H, H-4'), 4.33-4.36 (dd, 1H, *J* = 4.2 Hz and 10.8 Hz, H-5'a), 4.39 (t, 1H, *J* = 4.8 Hz, H-3'), 4.41-4.44 (dd, *J* = 3.0 Hz and 10.8 Hz, H-5'b), 4.66 (t, 1H, *J* = 4.8 Hz, H-2'), 6.07 (d, 1H, *J* = 4.8 Hz, H-1'), 8.21 (s, 1H, H-8), 8.29 (2, 1H, H-2); ^{13}C NMR (150 MHz, CD_3OD) δ 69.7 (C-5'), 71.9 (C-3'), 75.6 (C-2'), 83.7 (C-4'), 89.9 (C-1'), 120.4 (C-5), 140.9 (C-8), 150.7 (C-4), 154.0 (C-2), 157.4 (C-6); HRMS for $C_{10}H_{15}N_6O_6S$ ($[M+H]^+$) calcd: 347.0768 found 347.0767.

4.7.2 Model Building and analysis

Pdb entry 1c0a with the *E coli* Asp-tRNA synthetase was used as template. The aspartyl-adenosine-5'-monophosphate was remodeled into a sulfonamide with AspSoA **4.26** by substituting the phosphate group for a sulfonamide group as found in csd entry BIVTAB (without O5'). The Ade base and sugar are in the same position as in the original 1C0A structure. The Asp-sulfonamide is positioned by rotation of dihedral angles, so that the overlap with the original substrate in the X-ray structure is optimized (no clashes with the surrounding residues as verified by Chimera). Although the sulfonamide linker is shorter due to the missing O5', many of the original hydrogen bonds of the aspartyl-adenosine-5'-monophosphate with the enzyme are preserved upon substituting with the inhibitor in the active site.

4.7.3 Biological evaluation

4.7.3.1 Whole-cell activity determination

The respective bacteria were grown overnight in LB medium and cultured again the following day in fresh LB medium. Compounds were titrated in a 96-well plate using LB-medium to dilute the compounds. To each well, 85 µL LB-medium was added to a total volume of 90 µL. Next, 10 µL of bacterial cell culture grown to a OD_{600} of 0.1 was added. The cultures were next placed into a Tecan Infinite M200® incubator and shaken at 37°C, subsequently the OD_{600} was determined after 18 h. The broth dilution tests were performed in triplicates.

Bacterial strains used for the evaluations: *E. coli* K-12 BW28357, *S. aureus* (ATCC 6538), *S. lutea* (ATCC9341) and *C. albicans* CO11. The antibacterial activities of all compounds were determined by monitoring the optical density of suspensions of cell-cultures. All experiments have been performed in triplicate.

LB medium was prepared as follows: 10 g tryptone (Becton-Dickinson, cat. no. 211705), 5 g yeast extract (Becton-Dickinson, cat. no. 288620) and 10 g NaCl were dissolved in 800 mL of water. The pH was adjusted to 7.0 by 5N NaOH. The volume was adjusted to 1 L and the solution was sterilised.

4.7.3.2 Aminoacylation experiments:

These are as described previously in chapter 2, section 2.5.2.3.

5 General discussion

5.1 Background

In recent years, aaRSs have emerged as valuable and clinically validated targets for the development of anti-infective agents.[8] Among the several non-hydrolysable mimics of aa-AMP, aaSA analogues proved to be the strongest inhibitors of the corresponding aaRSs *in vitro*. However, these analogues can not be used as anti-infective agents due to their lack of selectivity and poor *in vivo* efficacy.[16] Efforts have been invested to improve the *in vivo* efficacy and selectivity of these analogues. For instance, in 1998, Cubist Pharmaceuticals reported a series of aaRS inhibitors where, although still based on aaSA analogues, the adenine base was replaced with an aryl-tetrazole moiety which was linked to the sugar through a two carbon linker. Although being strong and specific inhibitors of the corresponding aaRSs, these analogues could not be pursued further due to high serum albumin binding and poor cell penetration.[8, 61]

Previously, in our efforts to improve the *in vivo* efficacy of aaSA analogues by a Trojan-horse mechanism, aaSA analogues were coupled to the McC signal peptide and evaluated for their antibacterial activity. It has been found that these analogues also act as Trojan-horse inhibitors and their growth inhibitory properties were comparable to the parent McC. It has been shown that by replacing the *C*-terminal amino acid in McC analogues (the one which remains attached to the nucleoside moiety following metabolisation), different aaRSs could be targeted.[81] Different peptide variants of McC containing aaSAs as active moieties were synthesized and evaluated for their antibacterial activity. It has been likewise found that most of the alteration in the amino acid sequence had little effect on the uptake potential of these compounds whereas the *N*-formyl methionine at first position and the arginine at the second position are clearly essential for recognition by the YejABEF transporter.[82] In addition, the minimum peptide chain length of five amino acids is required for efficient uptake by the same transporter.[77] However, when aryl-tetrazole containing sulfamates were coupled to the McC signal peptide, these constructs failed to cross the cell membrane.[83] We therefore concluded that the YejABEF transporter may be selective for peptide-adenylates or very closely related derivatives (research work done by my colleague, Dr. G. Vondenhoff).

5.2 Siderophore-drug conjugates: an attempt to improve the *in vivo* efficacy

As our attempts to improve the *in vivo* efficacy of the aryl-terazole containing sulfamates by coupling them with the McC signal peptide were unsuccessful,[83] we looked for other transport systems such as an iron channel. Iron being an essential micronutrient required for all living organisms, it is a suitable candidate for a Trojan-horse strategy. Being selective inhibitors of the corresponding aaRSs, we opted for the aryl-tetrazole containing sulfamates instead of non-selective aaSA analogues. Out of several compounds reported by Cubist Pharmaceuticals, the compounds CB432 (**2.2**) and CB168 (**2.3**, chapter 2) were selected for their broad-spectrum of activity and high selectivity, respectively. We hypothesized that when a SDC-Fe(III) complex would be presented to bacteria, it will be internalized actively via their iron channel. Once inside the cell, the iron will be released by iron reductase and the SDC would be metabolized by broad-specificity peptidases to release the active moiety which will exert its antibacterial effect (analogous to McC). However, no antibacterial activity was observed for the trihydroxamate-based SDCs (**2.26** and **2.27**) although these SDCs did show nice *in vitro* activity in cell extracts (except ΔABN cell extracts which lack peptidase activity). Thus, the results indicate that these SDCs are efficiently metabolized by cellular peptidases and thus release the active moiety which exerts it inhibitory activity whereas intact SDC does not have an inhibitory effect on the amino acid incorporation. Hence, failure of uptake is the main reason for the inactivity of the SDCs. Therefore we concluded that analogous to the YejABEF transporter, the iron-channel may be selective for a peptide-adenylate construct or very closely related derivatives. However, the iron channel is a very complex system and comprised of FhuABCD proteins and an energy dependent TonB-ExbB-ExbD complex. Thus, at which stage the loss of the recognition resulted in impairment of the transport across the cell membrane remains to be determined. Along the same lines, we attempted to synthesize SDCs containing an aaSA as the active moiety. However, due to their chemical instability (concomitant formation of cycloadenosine) our efforts to synthesize the desired conjugates were unsuccessful (Scheme 2-4).

As the delivery mediated by trihydroxamate-based siderophores failed, we also looked for an unnatural and relatively simple siderophore such as the biscatecholate (**2.33**). This unnatural siderophore is known to promote the growth of several bacteria

indicating it can transport iron across the cell membrane.[115] Moreover, β-lactam antibiotics coupled to biscatecholate siderophores have shown before to enhance the antibacterial activity as of active transport via the iron channel.[110a] As catecholate siderophores use a different iron acquisition system, we assumed that iron acquisition may not be specific for peptide-adenylates. However, it was found that the biscatecholate-based SDC failed to release the active moiety. Recently, while studying intracellular processing of McC, it was uncovered that cellular peptidases could not metabolize the McC peptide if the *N*-terminal is protected or blocked with a formyl group. Moreover, these peptidases also lack endopeptidase activity. In other words, the intracellular peptidases metabolize the peptide sequentially from the *N*-terminal to the *C*-terminal and metabolisation is hampered if the *N*-terminal is protected.[122] In our SDC, the α-amine moiety of the ornithine is protected as a catecholamide which might be the limiting factor to release the active moiety. However, whether or not this SDC is recognized by the iron channel is less important as an *in vitro* experiment already showed lack of activity. In addition, our attempts to synthesize a linear enterobactin analogue were unsuccessful.

Overall, the choice of siderophore, linker and drug all seem to be very crucial for successful drug delivery. For successful siderophore mediated drug delivery, the SDC should be recognized by the iron channel and should undergo metabolism upon internalization to release the active moiety. While very few successful examples of SDCs have been reported in the literature, most of the SDCs having cytoplasmic targets showed lower activity compared to the free drugs. It appears that most often conjugation of the drug with a siderophore either perturbs its transport across the cell membrane or fails to release the drug.[105] As demonstrated by albomycin and salmycin, nature seems to have addressed these issues more cleverly. However, in case of salmycins, despite of good antibacterial activities in the *in vitro* assays, low *in vivo* activity was observed mainly due to chemical instability owing to its ester function connecting the siderophore and the active moiety.[99]

5.3 Base substituted 5'-*O*-(*N*-isoleucyl)sulfamoyl nucleoside analogues as potential antibacterial agents

When our attempts to improve the *in vivo* efficacy of aryl-tetrazole containing sulfamates were unsuccessful, we analyzed the structures of albomycin and trihydroxamate-based SDCs. This shows that albomycin carries a modified pyrimidine

attached to a thioxylofuranose, whereas our SDC has an aryl-tetrazole moiety connected to a ribose through a two carbon linker. Interestingly, both these analogues differ from aaSA analogues in having either a modified pyrimidine base or a heterocyclic base while still acting as potent and selective aaRS inhibitors. These observations prompted us to re-investigate the pharmacophoric importance of the adenine base in aaSA. Towards this end, we synthesized and evaluated several nucleoside sulfamate analogues containing natural and unnatural heterocyclic bases substituting for the adenine base. IleRS being an extensively studied enzyme before (e.g. mupirocin and the compounds reported by Cubist Pharmaceuticals all target IleRS) was chosen to study the effect of different bases substituting for adenine.

Several isoleucyl-sulfamoyl nucleosides hereto were synthesized and evaluated for their antibacterial activity. The remarkable order of inhibitory activity for the different base substituents found in the *in vitro* assays was U >I>A=C>4-ABI>4-NBI>G. Interestingly, U and C derivatives proved to be either more or equally active respectively, as compared to the original aaSA analogue. Although, the hypoxanthine derivative is well tolerated, additional substitution with a C-2 amino group as in the G analogue resulted in significant loss of the activity possibly due to steric clashes. Therefore we concluded that the adenine base is not a prerequisite for aaRS inhibition. However, no growth inhibitory activity was observed for different analogues up to 5 mM by the disc diffusion method. This lack of antibacterial activity was rather expected and can be contributed to poor cell penetration as found for the IleSA analogue. Therefore, with the McC strategy in mind, these analogues were coupled to the McC signal peptide in an effort to improve the uptake of these analogues via a Trojan-horse strategy. Unfortunately, only transient antibacterial activity was noticed for purine derivatives carrying the bases A, I or 4-ABI (1,3-dideazaadenine) following incubation with the Ara-Yej inducer strain. This observation again supports our previous assumption that the YejABEF transporter is selective for peptidyl-adenylate analogues or closely related derivatives. Apparently, even a conjugate carrying an unnatural base such as 1,3-dideazaadenine (4-ABI) can be transported via this YejABEF transporter. In addition, the lack of inhibitory activity found for G and 4-NBI conjugates can be explained from the marginal *in vitro* activity as seen for the guanosine derivative. Moreover, despite of excellent *in vitro* activities for the U and C derivatives, no growth inhibitory activity was observed for their hexapeptidyl

conjugates in a whole-cell assay. This lack of inhibitory activity can only be attributed to a lack of recognition at the transporter.

5.4 5'-(*N*-aminoacyl)-sulfonamido-5-deoxyadenosines: potentially stable alternative to aaSA analogues

As mentioned above, aaSA analogues could not be pursued as potential antibiotics due to their lack of selectivity and poor *in vivo* efficacy. Moreover, synthesis of aaSA analogues remained problematic due to the formation of cyacloadenosine as a side product. It has been reported in the literature that aaSA analogues are prone to form a cyclic degradation product (as shown in Scheme 2-4) resulting in low yields for coupling of an amino acid to the core and difficult purification.[114] Similarly, in our synthetic efforts to improve the *in vivo* efficacy of aaSA analogues by conjugation with a peptidic carrier (either siderophore or McC signal peptide), we consistently observed cycloadenosine as a side product. We failed to synthesize the desired siderophore conjugate of aaSA whereas synthesis of hexapeptidyl conjugates was low yielding with tedious purification.

In an effort to reduce this side reaction, we proposed 5'-(*N*-aminoacyl)-sulfonamido-5'-deoxyadenosine (aaSoA) as a potentially stable alternative to aaSA analogues. We hypothesized that deletion of the 5'-oxygen in aaSA analogues renders the C-5' less electrophilic which in turn should improve the stability of these analogues, while hopefully preserving equal potency as compared to aaSA analogues. We therefore synthesized several nucleoside sulfonamides and evaluated them for their inhibitory activity in *in vitro* and in whole-cell assays. It was shown however that these analogues (except for AspSoA) are not able to inhibit the corresponding aaRS. Only the AspSoA derivative displayed some growth inhibitory activity against *E. coli* wt whereas the intermediate sulfamate (SA analogue without any attached amino acid) showed a broad-spectrum of activity. As Asp-SoA also showed AspRS inhibition in the *in vitro* test, we assumed that this growth inhibitory effect against *E. coli* wt is mediated via AspRS inhibition. However, the mode of action of the SA intermediate remains to be determined.

There are several possible reasons for the loss of activity of these aaSoA derivatives as compared to their respective aaSA analogues. Most obvious reason could be the shortened overall length of the inhibitor which might impair H-bonding of the α-amino group of the inhibitor with the peptidic backbone of aaRS. Recently, we

established that the α-amino group is an important recognition point at the active site of aaRS. Alteration of the α-amino group may lead to complete loss of activity.[160] Alternatively, aaSoA analogues might fail to mimic the stereoelectronic properties of sulfamate. It has been observed previously that the distance between the sugar and the amino acid is important for tight binding. Alteration in the distance between the amino acid and the sugar resulted in significant loss of activity.[39c] However, whether it is the overall length or the charge around the sulfonamide linkage which contributes to the significant loss of affinity remains to be determined. Whether in the end these analogues are more stable as compared to the respective aaSA is less important now as the *in vitro* assays showed lack of activity against the respective aaRSs.

5.5 Future perspective

Although we have achieved limited success in promoting the *in vivo* efficacy of the aminoacyl-sulfamates, the results from this work can form a foundation on to which further development can be accomplished. As noted in the literature, release of the active moiety is always the main bottleneck in siderophore mediated drug delivery, which is attributed to stability of the linkage between the siderophore and the active moiety. Ester linkages are too labile, whereas some other linkages are too stable to release the active moiety. PepN contributes about 1% to the total intracellular protein concentration and is a major aminopeptidase. Chandu *et al.* demonstrated that pepN has endopeptidase activity and preferentially cleaves the peptide bond between the amino acids having either basic (Arg, Lys) or small (Gly, Ala) side chain.[162] Therefore, it is recommended that a dipeptide containing either combination of these four amino acids as a spacer could be inserted between the siderophore and the drug, so that selective endopeptidase activity by pepN can be exploited for releasing the active moiety (analogous to albomycin). Alternatively, one can use a danoxamine like siderophore where the SDC does not depend on any enzyme to release its active moiety. Here, upon iron removal, one of the *N*-hydroxyl groups act as a nucleophile to release the active moiety (Figure 1-11).

Therefore, it would be desirable to couple aryl-terazole containing sulfamates with different siderophores to find an ideal SDC which would fulfil all criteria as stated above. It was claimed that a mixed ligand siderophore containing SDC is able to use multiple iron-transport systems to deliver the active moiety.[108b] These mixed ligand siderophores (e.g. a combination of hydroxamate and catecholate component) were shown to be

promising candidates to obtain SDCs which would have a broad-spectrum of activity, being recognized by multiple iron-transport systems, but this research has not been followed up.

Moreover, the clinical use of albomycin is frequently advocated provided a sufficient quantity of albomycin can be isolated from producing strains or can be chemically synthesized. Benz *et al.* have synthesized an albomycin δ1 analogue substituting xylose for thioxylose but the compound was found to be inactive.[101] Synthetic efforts therefore need to achieve synthetic albomycin itself in sufficient quantities. Recently, albomycin biosynthetic genes have been identified that may pave the way to improve the production of albomycin itself or some biosynthetic intermediates by genetic engineering. The biosynthetic intermediates can be used to yield semisynthetic albomycin.[103] Synthesis of a modified serine having 4-thioxylofuranose substituents at the β-position is the rate limiting step in obtaining synthetic albomycin.

As different heterocyclic base substitutions for the adenine base are well tolerated, it would be interesting to evaluate such analogues for human IleRS inhibition. Being structurally different from the adenine base, some of these analogues have been shown to be selective for bacterial aaRSs over their human orthologs. Therefore, different heterocyclic modifications in aaSA analogues should be further explored to uncover aaRS inhibitors with excellent *in vivo* efficacy, high selectivity and better stability. In addition, different base substituted sulfamoyl nucleoside analogues could be coupled to a siderophore to improve their *in vivo* efficacy. These SDCs may yield some information about the structural requirement of the iron channel. As found for aaSoA analogues, eliminating the 5'-oxygen resulted in complete loss of activity (except for the AspSoA). Therefore, it would be interesting to synthesize and evaluate all remaining aminoacylated aaSoA analogues. Indeed, in analogy to AspSoA, deletion of the 5'-oxygen in aaSA derivatives may be well tolerated in other aaRS enzymes. Further detailed studies are needed to establish the SAR for these aaRS inhibitors. Along the same lines, to address the question whether it is the overall length or the charge around the sulfonamide linkage which is the main culprit for the loss binding affinity, it is desirable to synthesize and evaluate homoadenosine analogues replacing the 5'-oxygen with a methylene in aaSA. These analogues may rescue aaRS inhibitory activity owing to the same overall length as compared to aaSAs.

Nature has demonstrated that that a diverse set of chemicals can be used as aaRS inhibitors. Therefore, besides nucleoside antibiotics (like albomycin, McC, ascamycins) efforts should be invested to explore other natural compounds as a platform/lead for further optimization of structures, to yield aaRS inhibitors which could lead to clinically useful antibiotics. For example, indolmycin has a narrow spectrum of activity due to its hydrophobic nature.[42] More efforts could be invested to enhance the hydrophilicity of indolmycin and thus broaden the spectrum of activity. Recently, Anacor Pharmaceuticals elaborating on its boron-based compound platform reported substituted benzoxaborole derivatives (e.g. AN2690) for treatment of fungal infections. Unlike other aaRS inhibitors, AN2690 acts by inhibiting an editing site of LeuRS. This compound is in phase III clinical trials for treatment of onychomycosis.[9a] This confirms the editing site of aaRS could also be explored as a potential target. Therefore in general, random screening of heterocyclic compound libraries may yield aaRS inhibitors or lead compounds which could be further optimized to finally yield clinically useful antibiotics.

References

[1] (a)A. W. Maresso, O. Schneewind, *Pharmacol. Rev.* **2008**, *60*, 128-141; (b)K. Bockstael, A. Aerschot, *Cent. Eur. J. Med.* **2009**, *4*, 141-155.

[2] B. Spellberg, B. Taylor-Blake, *Infect. Dis. Poverty* **2013**, *2*, 3.

[3] *Removing obstacles to healthy development*, World Health Organization, **1999**.

[4] G. D. Wright, *Adv. Drug Delivery Rev.* **2005**, *57*, 1451-1470.

[5] (a)U. Möllmann, L. Heinisch, A. Bauernfeind, T. Köhler, D. Ankel-Fuchs, *Biometals* **2009**, *22*, 615-624; (b)S. Rachakonda, L. Cartee, *Curr. Med. Chem.* **2004**, *11*, 775-793.

[6] K. D. Krewulak, H. J. Vogel, *Biochim. Biophys. Acta, Biomembr.* **2008**, *1778*, 1781-1804.

[7] J. Pohlmann, *Drugs Fut.* **2004**, *29*, 243-251.

[8] P. Schimmel, J. Tao, J. Hill, *FASEB J.* **1998**, *12*, 1599-1609.

[9] (a)F. L. Rock, W. Mao, A. Yaremchuk, M. Tukalo, T. Crépin, H. Zhou, Y.-K. Zhang, V. Hernandez, T. Akama, S. J. Baker, J. J. Plattner, L. Shapiro, S. A. Martinis, S. J. Benkovic, S. Cusack, M. R. K. Alley, *Science* **2007**, *316*, 1759-1761; (b)P. R. Schimmel, D. Söll, *Annu. Rev. Biochem.* **1979**, *48*, 601-648; (c)M. Ibba, D. Söll, *Annu. Rev. Biochem.* **2000**, *69*, 617-650.

[10] (a)M. Ibba, A. W. Curnow, D. Söll, *Trends Biochem. Sci.* **1997**, *22*, 39-42; (b)W. F. Doolittle, J. R. Brown, *Proc. Nat. Acad. Sci. U.S.A.* **1994**, *91*, 6721-6728; (c)J. R. Brown, W. F. Doolittle, *Proc. Nat. Acad. Sci. U.S.A.* **1995**, *92*, 2441-2445.

[11] M. Delarue, *Curr. Opin. Struct. Biol.* **1995**, *5*, 48-55.

[12] G. H. M. Vondenhoff, A. Van Aerschot, *Eur. J. Med. Chem.* **2011**, *46*, 5227-5236.

[13] J. Tao, P. Wendler, G. Connelly, A. Lim, J. Zhang, M. King, T. Li, J. A. Silverman, P. R. Schimmel, F. P. Tally, *Proc. Nat. Acad. Sci. U.S.A.* **2000**, *97*, 783-786.

[14] G. Raczniak, M. Ibba, D. Söll, *Toxicol.* **2001**, *160*, 181-189.

[15] D. M. Bunk, J. J. Dalluge, M. J. Welch, *Anal. Biochem.* **2000**, *284*, 191-200.

[16] M. Ibba, C. Francklyn, S. Cusack, *The aminoacyl-tRNA synthetases*, Landes Bioscience Georgetown, TX, **2005**.

[17] J. Pohlmann, H. Brotz-Oesterhelt, *Curr. Drug Targets Infect. Disord.* **2004**, *4*, 261-272.

[18] (a)S. T. Rao, M. G. Rossmann, *J. Mol. Biol.* **1973**, *76*, 241-256; (b)G. Eriani, M. Delarue, O. Poch, J. Gangloff, D. Moras, *Nature* **1990**, *347*, 203-206.

[19] (a)B. Gadakh, A. Van Aerschot, *Expert. Opin. Ther. Pat.* **2012**, *22*, 1453-1465; (b)U. A. Ochsner, X. Sun, T. Jarvis, I. Critchley, N. Janjic, *Expert Opin. Investig. Drugs* **2007**, *16*, 573-593.

[20] (a)J. G. Hurdle, A. J. O'Neill, I. Chopra, *Antimicrob. Agents Chemother.* **2005**, *49*, 4821-4833; (b)S. Kim, S. W. Lee, E. C. Choi, S. Y. Choi, *Appl. Microbiol. Biotechnol.* **2003**, *61*, 278-288.

[21] R. Sankaranarayanan, A.-C. Dock-Bregeon, P. Romby, J. Caillet, M. Springer, B. Rees, C. Ehresmann, B. Ehresmann, D. Moras, *Cell* **1999**, *97*, 371-381.

[22] A. N. Baldwin, P. Berg, *J. Biol. Chem.* **1966**, *241*, 839-845.

[23] H. Jakubowski, E. Goldman, *Microbiol. Rev.* **1992**, *56*, 412-429.

[24] (a)A. J. Gale, J.-P. Shi, P. Schimmel, *Biochemistry* **1996**, *35*, 608-615; (b)M. Ibba, S. Sever, M. Praetorius-Ibba, D. Söll, *Nucleic Acids Res.* **1999**, *27*, 3631-3637.

[25] H. Ueda, Y. Shoku, N. Hayashi, J.-i. Mitsunaga, Y. In, M. Doi, M. Inoue, T. Ishida, *BBA. Protein Struct. M.* **1991**, *1080*, 126-134.

[26] A. R. Fersht, C. Dingwall, *Biochemistry* **1979**, *18*, 2627-2631.

[27] A. T. Norris, P. Berg, *Proc. Nat. Acad. Sci. U.S.A.* **1964**, *52*, 330-337.

[28] S. A. Martinis, M. T. Boniecki, *FEBS Lett.* **2010**, *584*, 455-459.

[29] A. Schon, C. G. Kannangara, S. Gough, D. Soll, *Nature* **1988**, *331*, 187-190.

[30] (a)M. Stanzel, A. SchÖN, M. Sprinzl, *Eur. J. Biochem.* **1994**, *219*, 435-439; (b)H. D. Becker, D. Kern, *Proc. Nat. Acad. Sci. U.S.A.* **1998**, *95*, 12832-12837; (c)S. Eiler, A. C. Dock-Bregeon, L. Moulinier, J. C. Thierry, D. Moras, *EMBO J.* **1999**, *18*, 6532-6541.

[31] (a)A. W. Curnow, D. L. Tumbula, J. T. Pelaschier, B. Min, D. Söll, *Proc. Nat. Acad. Sci. U.S.A.* **1998**, *95*, 12838-12843; (b)J. L. Huot, C. Balg, D. Jahn, J. Moser, A. Emond, S. P. Blais, R. Chenevert, J. Lapointe, *Biochemistry* **2007**, *46*, 13190-13198.
[32] D. L. Tumbula, H. D. Becker, W. Z. Chang, D. Soll, *Nature* **2000**, *407*, 106-110.
[33] C. Balg, M. D. Mieri, J. L. Huot, S. P. Blais, J. Lapointe, R. Chênevert, *Bioorg. Med. Chem.* **2010**, *18*, 7868-7872.
[34] (a)K. A. Pappa, *J. Am. Acad. Dermatol.* **1990**, *22*, 873-879; (b)E. B. Chain, G. Mellows, *J. Chem. Soc. Perkin Trans. 1* **1977**, 294-309.
[35] R. Sutherland, R. J. Boon, K. E. Griffin, P. J. Masters, B. Slocombe, A. R. White, *Antimicrob. Agents Chemother.* **1985**, *27*, 495-498.
[36] I. R. B. Hudson, *J. Hosp. Infect.* **1994**, *27*, 81-98.
[37] J. B. Patel, R. J. Gorwitz, J. A. Jernigan, *Clin. Infect. Dis.* **2009**, *49*, 935-941.
[38] J. E. Hodgson, S. P. Curnock, K. G. Dyke, R. Morris, D. R. Sylvester, M. S. Gross, *Antimicrob. Agents Chemother.* **1994**, *38*, 1205-1208.
[39] (a)A. K. Forrest, N. F. Osborne, D. Pengelly. Mupirocinsulfamates with antibacterial activity. WO005126A1, 1997; (b)I. Bennett, N. J. P. Broom, R. Cassels, J. S. Elder, N. D. Masson, P. J. O'Hanlon, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1847-1852; (c)M. J. B. Brown, L. M. Mensah, M. L. Doyle, N. J. P. Broom, N. Osbourne, A. K. Forrest, C. M. Richardson, P. J. O'Hanlon, A. J. Pope, *Biochemistry* **2000**, *39*, 6003-6011.
[40] U. Hornemann, L. H. Hurley, M. K. Speedie, H. G. Floss, *J. Am. Chem. Soc.* **1971**, *93*, 3028-3035.
[41] (a)R. G. Werner, L. F. Thorpe, W. Reuter, K. H. Nierhaus, *Eur. J. Biochem.* **1976**, *68*, 1-3; (b)M. Kitabatake, K. Ali, A. Demain, K. Sakamoto, S. Yokoyama, D. Söll, *J. Biol. Chem.* **2002**, *277*, 23882-23887.
[42] R. G. Werner, *Antimicrob. Agents Chemother.* **1980**, *18*, 858-862.
[43] R. G. Werner, A. L. Demain, *J. Antibiot. (Tokyo)* **1981**, *34*, 551-554.
[44] T. Kanamaru, Y. Nakano, Y. Toyoda, K.-I. Miyagawa, M. Tada, T. Kaisho, M. Nakao, *Antimicrob. Agents Chemother.* **2001**, *45*, 2455-2459.
[45] S. Vino, K. Lokesh, *Adv. Biotech.* **2008**, *6*, 22-26.
[46] M. S. Kuo, D. A. Yurek, D. A. Kloosterman, *J. Antibiot. (Tokyo)* **1989**, *42*, 1006-1007.
[47] B. Ruan, M. L. Bovee, M. Sacher, C. Stathopoulos, K. Poralla, C. S. Francklyn, D. Soll, *J. Biol. Chem.* **2005**, *280*, 571-577.
[48] (a)W. Paetz, G. Nass, *Eur. J. Biochem.* **1973**, *35*, 331-337; (b)G. Nass, K. Poralla, H. Zähner, *Biochem. Biophys. Res. Commun.* **1969**, *34*, 84-91; (c)S. C. Gerken, S. M. Arfin, *J. Biol. Chem.* **1984**, *259*, 9202-9206.
[49] (a)M. Konishi, M. Nishio, K. Saitoh, T. Miyaki, T. Oki, H. Kawaguchi, *J. Antibiot. (Tokyo)* **1989**, *42*, 1749-1755; (b)T. Iwamoto, E. Tsujii, M. Ezaki, A. Fujie, S. Hashimoto, M. Okuhara, M. Kohsaka, H. Imanaka, K. Kawabata, Y. Inamoto, et al., *J. Antibiot. (Tokyo)* **1990**, *43*, 1-7.
[50] J. O. Capobianco, D. Zakula, M. L. Coen, R. C. Goldman, *Biochem. Biophys. Res. Commun.* **1993**, *190*, 1037-1044.
[51] J. Mittendorf, F. Kunisch, M. Matzke, H.-C. Militzer, A. Schmidt, W. Schönfeld, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 433-436.
[52] (a)R. Petraitiene, V. Petraitis, A. M. Kelaher, A. A. Sarafandi, D. Mickiene, A. H. Groll, T. Sein, J. Bacher, T. J. Walsh, *Antimicrob. Agents Chemother.* **2005**, *49*, 2084-2092; (b)A. Hasenoehrl, T. Galić, G. Ergović, N. Maršić, M. Skerlev, J. Mittendorf, U. Geschke, A. Schmidt, W. Schoenfeld, *Antimicrob. Agents Chemother.* **2006**, *50*, 3011-3018.
[53] D. Hoepfner, Case W. McNamara, Chek S. Lim, C. Studer, R. Riedl, T. Aust, Susan L. McCormack, David M. Plouffe, S. Meister, S. Schuierer, U. Plikat, N. Hartmann, F. Staedtler, S. Cotesta, Esther K. Schmitt, F. Petersen, F. Supek, Richard J. Glynne, John A. Tallarico, Jeffrey A. Porter, Mark C. Fishman, C. Bodenreider, Thierry T. Diagana, N. R. Movva, Elizabeth A. Winzeler, *Cell Host & Microbe* **2012**, *11*, 654-663.

[54] (a)S. F. Ataide, M. Ibba, *ACS Chem. Biol.* **2006**, *1*, 285-297; (b)J. G. Kim, B. K. Park, S. U. Kim, D. Choi, B. H. Nahm, J. S. Moon, J. S. Reader, S. K. Farrand, I. Hwang, *Proc. Nat. Acad. Sci. U S A* **2006**, *103*, 8846-8851; (c)J. S. Reader, P. T. Ordoukhanian, J. G. Kim, V. de Crecy-Lagard, I. Hwang, S. Farrand, P. Schimmel, *Science* **2005**, *309*, 1533.

[55] (a)M. Ubukata, H. Osada, J. Magae, K. Isono, *Agric. Biol. Chem.* **1988**, *52*, 1117-1122; (b)H. Osada, K. Isono, *Antimicrob. Agents Chemother.* **1985**, *27*, 230-233.

[56] M. J. Brown, P. S. Carter, A. E. Fenwick, A. P. Fosberry, D. W. Hamprecht, M. J. Hibbs, R. L. Jarvest, L. Mensah, P. H. Milner, P. J. O'Hanlon, A. J. Pope, C. M. Richardson, A. West, D. R. Witty, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3171-3174.

[57] (a)P. Brown, C. M. Richardson, L. M. Mensah, P. J. O'Hanlon, N. F. Osborne, A. J. Pope, G. Walker, *Bioorg. Med. Chem.* **1999**, *7*, 2473-2485; (b)S. Bernier, D. Y. Dubois, M. Therrien, J. Lapointe, R. Chenevert, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2441-2444.

[58] (a)J. Lee, S. U. Kang, M. K. Kang, M. W. Chun, Y. J. Jo, J. H. Kkwak, S. Kim, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1365-1370; (b)J. Lee, S. U. Kang, S. Y. Kim, S. E. Kim, M. K. Kang, Y. J. Jo, S. Kim, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 961-964; (c)J. Lee, S. U. Kang, S. Y. Kim, S. E. Kim, Y. J. Jo, S. Kim, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 965-968.

[59] (a)D. Heacock, C. J. Forsyth, K. Shiba, K. Musier-Forsyth, *Bioorg. Chem.* **1996**, *24*, 273-289; (b)H. Belrhali, A. Yaremchuk, M. Tukalo, K. Larsen, C. Berthet-Colominas, R. Leberman, B. Beijer, B. Sproat, J. Als-Nielsen, G. Grubel, a. et, *Science* **1994**, *263*, 1432-1436.

[60] J. Lee, S. E. Kim, J. Y. Lee, S. Y. Kim, S. U. Kang, S. H. Seo, M. W. Chun, T. Kang, S. Y. Choi, H. O. Kim, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1087-1092.

[61] J. Hill, G. Yu, Y.-K. Shue, T. M. Zydowsky, J. Rebek. Aminoacyl adenylate mimics as novel antimicrobial and antiparasitic agents. 5726195, 1998.

[62] I. A. Critchley, L. S. Green, C. L. Young, J. M. Bullard, R. J. Evans, M. Price, T. C. Jarvis, J. W. Guiles, N. Janjic, U. A. Ochsner, *J. Antimicrob. Chemother.* **2009**, *63*, 954-963.

[63] S. Shibata, J. R. Gillespie, A. M. Kelley, A. J. Napuli, Z. Zhang, K. V. Kovzun, R. M. Pefley, J. Lam, F. H. Zucker, W. C. Van Voorhis, E. A. Merritt, W. G. J. Hol, C. L. M. J. Verlinde, E. Fan, F. S. Buckner, *Antimicrob. Agents Chemother.* **2011**, *55*, 1982-1989.

[64] S. Shibata, J. R. Gillespie, R. M. Ranade, C. Y. Koh, J. E. Kim, J. U. Laydbak, F. H. Zucker, W. G. J. Hol, C. L. M. J. Verlinde, F. S. Buckner, E. Fan, *J. Med. Chem.* **2012**, *55*, 6342-6351.

[65] (a)S. J. Baker, T. Akama, B. K. Carolyn, V. Hernandez, K. M. Hold, J. J. Leyden, K. R. Maples, J. J. Plattner, V. Sander, Y.-K. Zhang. Boron containing small molecules. US7582621B2, 2009; (b)S. J. Baker, T. Akama, B. K. Carolyn, V. Hernandez, K. M. Hold, J. J. Leyden, K. R. Maples, J. Plattner, V. Sander, Y.-K. Zhang. Boron containing small molecules. WO0234981A1, 2006; (c)S. J. Baker, T. Akama, M. R. K. Alley, S. J. Benkovic, M. DiPierro, V. S. Hernandez, K. M. Hold, I. Kennedy, I. Likhotvorik, W. Mao, K. R. Maples, J. Plattner, F. Rock, V. Sanders, A. M. Stemphoski, G. P. Yiannikouros, S. Zegar, Y.-K. Zhang, H. Zhou. Boron containing small molecules. US8115026B2, 2012; (d)S. J. Baker, T. Akama, B. K. Carolyn, V. Hernandez, K. M. Hold, J. J. Leyden, K. R. Maples, J. J. Plattner, V. Sander, Y.-K. Zhang. Boron containing small molecules. US8039451B2, 2011.

[66] (a)K. Severinov, S. K. Nair, *Future Microbiol.* **2012**, *7*, 281-289; (b)F. Moreno, J. E. Gónzalez-Pastor, M.-R. Baquero, D. Bravo, *Biochimie* **2002**, *84*, 521-529.

[67] R. Kolter, F. Moreno, *Annu. Rev. Microbiol.* **1992**, *46*, 141-161.

[68] S. Duquesne, V. Petit, J. Peduzzi, S. Rebuffat, *J. Mol. Microbiol. Biotechnol.* **2007**, *13*, 200-209.

[69] D. Destoumieux-Garzón, J. Peduzzi, S. Rebuffat, *Biochimie* **2002**, *84*, 511-519.

[70] J. I. Guijarro, J. E. González-Pastor, F. Baleux, J. L. S. Millán, M. A. Castilla, M. Rico, F. Moreno, M. Delepierre, *J. Biol. Chem.* **1995**, *270*, 23520-23532.

[71] I. A. Khmel, V. M. Bondarenko, I. M. Manokhina, E. I. Basyuk, A. Z. Metlitskaya, V. A. Lipasova, Y. M. Romanova, *FEMS Microbiol. Lett.* **1993**, *111*, 269-274.
[72] D. E. Fomenko, A. Z. Metlitskaya, J. Péduzzi, C. Goulard, G. S. Katrukha, L. V. Gening, S. Rebuffat, I. A. Khmel, *Antimicrob. Agents Chemother.* **2003**, *47*, 2868-2874.
[73] (a)F. Baquero, D. Bouanchaud, M. C. Martinez-Perez, C. Fernandez, *J. Bacteriol.* **1978**, *135*, 342-347; (b)K. Severinov, S. K. Nair, *Future microbiology* **2012**, *7*, 281-289.
[74] R. F. Roush, E. M. Nolan, F. Löhr, C. T. Walsh, *J. Am. Chem. Soc.* **2008**, *130*, 3603-3609.
[75] A. Metlitskaya, T. Kazakov, G. H. Vondenhoff, M. Novikova, A. Shashkov, T. Zatsepin, E. Semenova, N. Zaitseva, V. Ramensky, A. Van Aerschot, K. Severinov, *J. Bacteriol.* **2009**, *191*, 2380-2387.
[76] S. M. Eswarappa, K. K. Panguluri, M. Hensel, D. Chakravortty, *Microbiology* **2008**, *154*, 666-678.
[77] G. H. M. Vondenhoff, B. Blanchaert, S. Geboers, T. Kazakov, K. A. Datsenko, B. L. Wanner, J. Rozenski, K. Severinov, A. Van Aerschot, *J. Bacteriol.* **2011**, *193*, 3618-3623.
[78] A. Metlitskaya, T. Kazakov, A. Kommer, O. Pavlova, M. Praetorius-Ibba, M. Ibba, I. Krasheninnikov, V. Kolb, I. Khmel, K. Severinov, *J. Biol. Chem.* **2006**, *281*, 18033-18042.
[79] M. Novikova, T. Kazakov, G. H. Vondenhoff, E. Semenova, J. Rozenski, A. Metlytskaya, I. Zukher, A. Tikhonov, A. Van Aerschot, K. Severinov, *J. Biol. Chem.* **2010**, *285*, 12662-12669.
[80] A. Tikhonov, T. Kazakov, E. Semenova, M. Serebryakova, G. Vondenhoff, A. Van Aerschot, J. S. Reader, V. M. Govorun, K. Severinov, *J. Biol. Chem.* **2010**, *285*, 37944-37952.
[81] G. H. Vondenhoff, S. Dubiley, K. Severinov, E. Lescrinier, J. Rozenski, A. Van Aerschot, *Bioorg. Med. Chem.* **2011**, *19*, 5462-5467.
[82] P. Van de Vijver, G. H. Vondenhoff, T. S. Kazakov, E. Semenova, K. Kuznedelov, A. Metlitskaya, A. Van Aerschot, K. Severinov, *J. Bacteriol.* **2009**, *191*, 6273-6280.
[83] G. H. Vondenhoff, B. Gadakh, K. Severinov, A. Van Aerschot, *Chembiochem : a European journal of chemical biology* **2012**, *13*, 1959-1969.
[84] R. Wise, T. Hart, O. Cars, M. Streulens, R. Helmuth, P. Huovinen, M. Sprenger, *Br. Med. J.* **1998**, *317*, 609-610.
[85] K. N. Raymond, C. J. Carrano, *Acc. Chem. Res.* **1979**, *12*, 183-190.
[86] (a)B. C. Chu, A. Garcia-Herrero, T. H. Johanson, K. D. Krewulak, C. K. Lau, R. S. Peacock, Z. Slavinskaya, H. J. Vogel, *Biometals* **2010**, *23*, 601-611; (b)M. Miethke, M. A. Marahiel, *Microbiol. Mol. Biol. Rev.* **2007**, *71*, 413-451.
[87] G. Bernier, V. Girijavallabhan, A. Murray, N. Niyaz, P. Ding, M. J. Miller, F. Malouin, *Antimicrob. Agents Chemother.* **2005**, *49*, 241-248.
[88] R. J. Abergel, A. M. Zawadzka, T. M. Hoette, K. N. Raymond, *J. Am. Chem. Soc.* **2009**, *131*, 12682-12692.
[89] M. J. Miller, F. Malouin, *Acc. Chem. Res.* **1993**, *26*, 241-249.
[90] M. J. Miller, *Chem. Rev.* **1989**, *89*, 1563-1579.
[91] (a)R. J. Bergeron, *Chem. Rev.* **1984**, *84*, 587-602; (b)R. C. Hider, X. Kong, *Nat. Prod. Rep.* **2010**, *27*, 637-657; (c)V. Braun, *J. Bacteriol.* **2009**, *191*, 3431-3436.
[92] (a)R. V. Somu, H. Boshoff, C. Qiao, E. M. Bennett, C. E. Barry, 3rd, C. C. Aldrich, *J. Med. Chem.* **2006**, *49*, 31-34; (b)R. V. Somu, D. J. Wilson, E. M. Bennett, H. I. Boshoff, L. Celia, B. J. Beck, C. E. Barry, 3rd, C. C. Aldrich, *J. Med. Chem.* **2006**, *49*, 7623-7635; (c)J. A. Ferreras, J. S. Ryu, F. Di Lello, D. S. Tan, L. E. Quadri, *Nat. Chem. Biol.* **2005**, *1*, 29-32.
[93] G. Gause, *Br. Med. J.* **1955**, *2*, 1177-1179.
[94] (a)G. Benz, T. Schröder, J. Kurz, C. Wünsche, W. Karl, G. Steffens, J. Pfitzner, D. Schmidt, *Angew. Chem. Int. Ed.* **1982**, *21*, 1322-1335; (b)G. Benz, *Liebigs Annalen der Chemie* **1984**, *1984*, 1399-1407; (c)G. Benz, L. Born, M. Brieden, R. Grosser, J. Kurz, H.

Paulsen, V. Sinnwell, B. Webber, *Liebigs Annalen der Chemie* **1984**, *1984*, 1408-1423; (d)G. Benz, *Liebigs Annalen der Chemie* **1984**, *1984*, 1424-1433.
[95] (a)Y. Zeng, H. Roy, P. B. Patil, M. Ibba, S. Chen, *Antimicrob. Agents Chemother.* **2009**, *53*, 4619-4627; (b)A. Pramanik, U. H. Stroeher, J. Krejci, A. J. Standish, E. Bohn, J. C. Paton, I. B. Autenrieth, V. Braun, *Int. J. Med. Microbiol.* **2007**, *297*, 459-469.
[96] A. Pramanik, V. Braun, *J. Bacteriol.* **2006**, *188*, 3878-3886.
[97] A. D. Ferguson, V. Braun, H. P. Fiedler, J. W. Coulton, K. Diederichs, W. Welte, *Protein Sci.* **2000**, *9*, 956-963.
[98] V. Braun, K. Günthner, K. Hantke, L. Zimmermann, *J. Bacteriol.* **1983**, *156*, 308-315.
[99] V. Braun, A. Pramanik, T. Gwinner, M. Köberle, E. Bohn, *Biometals* **2009**, *22*, 3-13.
[100] G. Schultz-Hauser, W. Köster, H. Schwarz, V. Braun, *J. Bacteriol.* **1992**, *174*, 2305-2311.
[101] H. Paulsen, M. Brieden, G. Benz, *Liebigs Annalen der Chemie* **1987**, *1987*, 565-575.
[102] Y.-M. Lin, M. J. Miller, *J. Org. Chem.* **1999**, *64*, 7451-7458.
[103] Y. Zeng, A. Kulkarni, Z. Yang, P. B. Patil, W. Zhou, X. Chi, S. Van Lanen, S. Chen, *ACS Chem. Biol.* **2012**, *7*, 1565-1575.
[104] (a)L. Dong, J. M. Roosenberg, M. J. Miller, *J. Am. Chem. Soc.* **2002**, *124*, 15001-15005; (b)L. Vértesy, W. Aretz, H.-W. Fehlhaber, H. Kogler, *Helvetica Chim. Acta* **1995**, *78*, 46-60.
[105] T. Wencewicz, U. Möllmann, T. Long, M. Miller, *Biometals* **2009**, *22*, 633-648.
[106] J. M. Roosenberg, 2nd, M. J. Miller, *J. Org. Chem.* **2000**, *65*, 4833-4838.
[107] H. Bickel, P. Mertens, V. Prelog, J. Seibl, A. Walser, *Tetrahedron* **1966**, *22, Supplement 8*, 171-179.
[108] (a)J. M. Roosenberg Ii, Y. M. Lin, Y. Lu, M. J. Miller, *Curr. Med. Chem.* **2000**, *7*, 159-197; (b)A. Ghosh, M. Ghosh, C. Niu, F. Malouin, U. Moellmann, M. J. Miller, *Chem. Biol.* **1996**, *3*, 1011-1019.
[109] C. Ji, P. A. Miller, M. J. Miller, *J. Am. Chem. Soc.* **2012**, *134*, 9898-9901.
[110] (a)S. Wittmann, M. Schnabelrauch, I. Scherlitz-Hofmann, U. Mollmann, D. Ankel-Fuchs, L. Heinisch, *Bioorg. Med. Chem.* **2002**, *10*, 1659-1670; (b)L. Heinisch, S. Wittmann, T. Stoiber, A. Berg, D. Ankel-Fuchs, U. Mollmann, *J. Med. Chem.* **2002**, *45*, 3032-3040.
[111] Y. Fakuda, *Drugs Fut.* **2009**, *34*, 127-136.
[112] R. L. Jarvest, J. M. Berge, P. Brown, D. W. Hamprecht, D. J. McNair, L. Mensah, P. J. O'Hanlon, A. J. Pope, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 715-718.
[113] (a)E. Masiukiewicz, S. Wiejak, B. Rzeszotarskat, *Org. Prep. Proced. Int.* **2002**, *34*, 531-537; (b)S. Wiejak, E. Masiukiewicz, B. Rzeszotarsk, *Chemical & pharmaceutical bulletin* **2001**, *49*, 1189-1191.
[114] (a)I. N. Redwan, T. Ljungdahl, M. Grøtli, *Tetrahedron* **2012**, *68*, 1507-1514; (b)P. Van de Vijver, T. Ostrowski, B. Sproat, J. Goebels, O. Rutgeerts, A. Van Aerschot, M. Waer, P. Herdewijn, *J. Med. Chem.* **2008**, *51*, 3020-3029; (c)E. M. Peterson, J. Brownell, R. Vince, *J. Med. Chem.* **1992**, *35*, 3991-4000.
[115] M. Schnabelrauch, S. Wittmann, K. Rahn, U. Mollmann, R. Reissbrodt, L. Heinisch, *Biometals* **2000**, *13*, 333-348.
[116] I. G. O'Brien, F. Gibson, *Biochim. Biophys. Acta* **1970**, *215*, 393-402.
[117] J. R. Pollack, J. B. Neilands, *Biochem. Biophys. Res. Commun.* **1970**, *38*, 989-992.
[118] A. Avdeef, S. R. Sofen, T. L. Bregante, K. N. Raymond, *J. Am. Chem. Soc.* **1978**, *100*, 5362-5370.
[119] W. H. Rastetter, T. J. Erickson, M. C. Venuti, *J. Org. Chem.* **1981**, *46*, 3579-3590.
[120] (a)S. R. Chhabra, A. Mahajan, W. C. Chan, *J. Org. Chem.* **2002**, *67*, 4017-4029; (b)R. Andruszkiewicz, D. Rożkiewicz, *Synth. Commun.* **2004**, *34*, 1049-1056.
[121] Z. Zhang, A. V. Aerschot, C. Hendrix, R. Busson, F. David, P. Sandra, P. Herdewijn, *Tetrahedron* **2000**, *56*, 2513-2522.
[122] T. Kazakov, G. H. Vondenhoff, K. A. Datsenko, M. Novikova, A. Metlitskaya, B. L. Wanner, K. Severinov, *J. Bacteriol.* **2008**, *190*, 2607-2610.

[123] B. E. Allred, C. Correnti, M. C. Clifton, R. K. Strong, K. N. Raymond, *ACS Chem. Biol.* **2013**, *8*, 1882-1887.
[124] L.-h. Zhang, G. S. Kauffman, J. A. Pesti, J. Yin, *J. Org. Chem.* **1997**, *62*, 6918-6920.
[125] I. W. Harvey, M. D. McFarlane, D. J. Moody, D. M. Smith, *J. Chem. Soc. Perkin Trans. 1* **1988**, 1939-1943.
[126] E. Sawicki, A. Carr, *J. Org. Chem.* **1957**, *22*, 503-506.
[127] J. A. Elvidge, G. T. Newbold, A. Percival, I. R. Senciall, *J. Chem. Soc. (Resumed)* **1965**, 5119-5120.
[128] T. A. Devlin, D. J. Jebaratnam, *Synthetic Commun.* **1995**, *25*, 711-718.
[129] T. Nakama, O. Nureki, S. Yokoyama, *J. Biol. Chem.* **2001**, *276*, 47387-47393.
[130] Y. Zhang, *BMC Bioinf.* **2008**, *9*, 40.
[131] M. Rance, O. W. Sorensen, G. Bodenhausen, G. Wagner, R. R. Ernst, K. Wuthrich, *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479-485.
[132] J. Schleucher, M. Schwendinger, M. Sattler, P. Schmidt, O. Schedletzky, S. J. Glaser, O. W. Sørensen, C. Griesinger, *J Biomol NMR* **1994**, *4*, 301-306.
[133] J. Ruiz-Cabello, G. W. Vuister, C. T. Moonen, P. Van Gelderen, J. S. Cohen, P. Van Zijl, *J. Magnetic Resonance (1969)* **1992**, *100*, 282-302.
[134] (a)L. Lain, H. Lönnberg, T. Lönnberg, *Chem. A Eur. J.* **2013**, *19*, 12424-12434; (b)Y. Hisamatsu, K. Hasada, F. Amano, Y. Tsubota, Y. Wasada-Tsutsui, N. Shirai, S.-i. Ikeda, K. Odashima, *Chem. A Eur. J.* **2006**, *12*, 7733-7741.
[135] F. W. Kotch, V. Sidorov, Y.-F. Lam, K. J. Kayser, H. Li, M. S. Kaucher, J. T. Davis, *J. Am. Chem. Soc.* **2003**, *125*, 15140-15150.
[136] H. P. Kokatla, M. K. Lakshman, in *Curr. Protoc. Nucleic Acid Chem.*, John Wiley & Sons, Inc., **2001**.
[137] X.-F. Zhu, H. J. Williams, A. I. Scott, *J. Chem. Soc., Perkin Trans. 1* **2000**, 2305-2306.
[138] J. Ling, K. M. Peterson, I. Simonović, C. Cho, D. Söll, M. Simonović, *Proc. Nat. Acad. Sci. U.S.A.* **2012**, *109*, 3281-3286.
[139] L. Holm, C. Sander, *Science* **1996**, *273*, 595-602.
[140] R. Salomon-Ferrer, D. A. Case, R. C. Walker, *WIREs Comput. Mol. Sci.* **2013**, *3*, 198-210.
[141] A. Pérez, I. Marchán, D. Svozil, J. Sponer, T. E. Cheatham Iii, C. A. Laughton, M. Orozco, *Biophysical J.* **2007**, *92*, 3817-3829.
[142] J. Wang, R. M. Wolf, J. W. Caldwell, P. A. Kollman, D. A. Case, *J. Comput. Chem.* **2004**, *25*, 1157-1174.
[143] P. A. Kollman, I. Massova, C. Reyes, B. Kuhn, S. Huo, L. Chong, M. Lee, T. Lee, Y. Duan, W. Wang, O. Donini, P. Cieplak, J. Srinivasan, D. A. Case, T. E. Cheatham, *Acc. Chem. Res.* **2000**, *33*, 889-897.
[144] E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, T. E. Ferrin, *J. Comput. Chem.* **2004**, *25*, 1605-1612.
[145] A. C. Wallace, R. A. Laskowski, J. M. Thornton, *Protein Eng.* **1995**, *8*, 127-134.
[146] J. Tao, P. Schimmel, *Expert Opin. Invest. Drugs* **2000**, *9*, 1767-1775.
[147] P. Van de Vijver, G. H. Vondenhoff, S. Denivelle, J. Rozenski, J. Verhaegen, A. Van Aerschot, P. Herdewijn, *Bioorg. Med. Chem.* **2009**, *17*, 260-269.
[148] C. T. Supuran, A. Casini, A. Scozzafava, *Med Res. Rev.* **2003**, *23*, 535-558.
[149] S. Nada, P. K. Chang, J. D. Dignam, *J. Biol. Chem.* **1993**, *268*, 7660-7667.
[150] (a)J. Perruchon, R. Ortmann, M. Schlitzer, *Synthesis* **2007**, *2007*, 3553-3557; (b)A. J. Brouwer, R. M. J. Liskamp, *Synlett* **2011**, *2011*, 2228-2230; (c)H. H. Bosshard, R. Mory, M. Schmid, H. Zollinger, *Helvetica Chimica Acta* **1959**, *42*, 1653-1658.
[151] (a)J. Huang, T. S. Widlanski, *Tetrahedron Lett.* **1992**, *33*, 2657-2660; (b)V. c. Ulgar, I. Maya, J. Fuentes, J. G. Fernández-Bolaños, *Tetrahedron* **2002**, *58*, 7967-7973.
[152] (a)G. Blotny, *Tetrahedron Lett.* **2003**, *44*, 1499-1501; (b)K. Venkataraman, D. R. Wagle, *Tetrahedron Lett.* **1979**, *20*, 3037-3040.
[153] T. Kataoka, T. Iwama, T. Setta, A. Takagi, *Synthesis* **1998**, *1998*, 423-426.

[154] S. Critchley, T. G. Gant, S. P. Langston, E. J. Olhava, S. Peluso. Substituted pyrrolo[2,3-d]pyrimidines as inhibitors of E1 activating enzymes. 2011.
[155] M. Pignot, G. Pljevaljcic, E. Weinhold, *E. J. Org. Chem.* **2000**, *2000*, 549-555.
[156] S. Hanashima, Y. Mizushina, T. Yamazaki, K. Ohta, S. Takahashi, H. Sahara, K. Sakaguchi, F. Sugawara, *Bioorg. Med. Chem.* **2001**, *9*, 367-376.
[157] Y.-M. Pu, A. Christesen, Y.-Y. Ku, *Tetrahedron Lett.* **2010**, *51*, 418-421.
[158] J. L. Anglin, L. Deng, Y. Yao, G. Cai, Z. Liu, H. Jiang, G. Cheng, P. Chen, S. Dong, Y. Song, *J. Med. Chem.* **2012**, *55*, 8066-8074.
[159] (a)J. Castro-Pichel, M. T. García-López, F. G. De las Heras, *Tetrahedron* **1987**, *43*, 383-389; (b)M. Okada, S. Iwashita, N. Koizumi, *Tetrahedron Lett.* **2000**, *41*, 7047-7051; (c)J. J. Fleming, K. W. Fiori, J. Du Bois, *J. Am. Chem. Soc.* **2003**, *125*, 2028-2029.
[160] G. H. Vondenhoff, K. Pugach, B. Gadakh, L. Carlier, J. Rozenski, M. Froeyen, K. Severinov, A. Van Aerschot, *PLoS ONE* **2013**, *8*, e79234.
[161] (a)A. P. Townsend, S. Roth, H. E. L. Williams, E. Stylianou, N. R. Thomas, *Org. Lett.* **2009**, *11*, 2976-2979; (b)Y. Xu, H. Jin, Z. Yang, L. Zhang, L. Zhang, *Tetrahedron* **2009**, *65*, 5228-5239.
[162] D. Chandu, D. Nandi, *Microbiology* **2003**, *149*, 3437-3447.

Curriculum vitae

Bharat Gadakh was born in 1984 in Thangaon (Sinnar), India. He commenced his bachelor studies at the University of Pune in 2002 where he obtained his bachelor in Pharmacy in 2006. He then moved to the National Instituite of Pharmaceutical Education and Research (NIPER), Mohali, India where he obtained his M.S. (Pharm) in Medicinal Chemistry with cGPA of 9.74 on a 10 point scale. He also worked as Junior Research Fellow at the Department of Chemistry at the Indian Institute of Technology, Delhi from 2008 to 2010. During the same year he started his PhD studies at the Laboratory of Medicinal Chemistry, Rega Institute for Medical Research, KU Leuven under the supervision Prof. Dr. Arthur Van Aerschot.

PUBLICATIONS AND REVIEWS

1. **Bharat K. Gadakh**, Premanand R. Patil, Satish Malik and K. P. Ravindranathan Kartha* "Novel selectivity in carbohydrate reactions, IV: DABCO mediated regioselective primary hydroxyl protection of carbohydrates." *Synthetic Communications*, **2009,** *39*, p- 2430-2438**.**
2. V. Haridas*, Yogesh K. Sharma, Srikanta Sahu, Ram P. Verma, Sandhya Sadanandan, **Bharat K. Gadakh**, "Designer peptide dendrimers using click reaction." *Tetrahedron*, **2011**, *67*(10), 1873-1884.

Thesis work;

1. Gaston Vondenhoff[¤], **Bharat Gadakh**[¤], Konstantin Severinov and Arthur Van Aerschot, "Microcin C and albomycin analogues with aryl-tetrazole substituents as nucleobase isosters are selective inhibitors of bacterial aminoacyl tRNA synthetases but lack efficient uptake." *Chembiochem,* **2012**, *13*(13), p-1959-1969. (**¤ equal contribution**).
2. **Bharat Gadakh** and Arthur Van Aerschot*, "Aminoacyl-tRNA synthetase inhibitors as antimicrobial agents: A patent review from 2006 till present" *Expert Opinion on Therapeutic Patents*, **2012**, *22*(12), p-1453-1465.
3. Gaston H. Vondenhoff, Ksenia Pougach, **Bharat Gadakh**, Laurence Carlier, Jef Rozenski, Mathy Froeyen, Konstantin Severinov and Arthur Van Aerschot*, "*N*-alkylated aminoacyl sulfamoyladenosines as potential inhibitors of aminoacylation

reactions and microcin C analogues containing D-amino acids" **2013**, PLoS ONE 8(11): e79234. doi:10.1371/journal.pone.0079234.

4. **Bharat Gadakh,** Gaston Vondenhoff, Eveline Lescrinier, Mathy Froyen and Arthur Van Aerschot* "5'-*O*-(*N*-L-isoleucyl) sulfamoyl nucleoside as potential antibacterial agents." *Bioorg. Med. Chem.,* **2014**, *22*(10), p-2875-2886.
5. Agarwal Vinayak, Vondenhoff Gaston, **Bharat Gadakh,** Severinov Konstantin, Van Aerschot Arthur, Nair, Satish*, "Exploring the substrate promiscuity of an antibiotic inactivating enzyme" *Medchemcomm, under review.*
6. **Bharat Gadakh,** Gaston Vondenhoff, Simon Smaers and Arthur Van Aerschot* "5'-(*N*-L-aminoacyl-sulfonamido)-5'-deoxyadenosine: A more stable alternative to aminoacyl-sulfamoyl adenosines?" *manuscript under preparation.*

Conference and summer school attended (ppresentation)

1. **Bharat K. Gadakh**, Gaston Vondenhoff and Arthur Van Aerschot*, "A Trojan-horse strategy for antimicrobial therapy." The 15th Sigma-Aldrich Organic Synthesis Meeting at Spa, 1st and 2nd Dec, 2011.
2. **Bharat K. Gadakh**, Gaston Vondenhoff and Arthur Van Aerschot*, "Siderophore-drug conjugates: An attempt to improve *in vivo* efficacy of 5'*O*-(*N*-aminoacyl) sulfamoyl adenosines." First spring symposium, Leuven, on 14th March, 2012.
3. **Bharat K. Gadakh**, Gaston Vondenhoff and Arthur Van Aerschot*, "Siderophore-drug conjugates: a tool to improve *in vivo* efficacy of antibacterial agents." The 16th Sigma-Aldrich Organic Synthesis Meeting at Spa, 6th and 7th Dec, 2012.
4. **Bharat K. Gadakh,** Gaston Vondenhoff, Eveline Lescrinier and Arthur Van Aerschot*, "Design, synthesis and biological evaluation of nucleoside sulfamate as isoleucyl tRNA synthetase inhibitors." 5th European Conference on Chemistry for Life Sciences (5th ECCLS), Barcelona, 10-12th June, 2013.
5. **Bharat K. Gadakh,** Gaston Vondenhoff, Eveline Lescrinier and Arthur Van Aerschot*, "5'-*O*-(*N*-aminoacyl)-sulfamoyl nucleoside analogues as potential antibacterial agents." ULLA Summer School, UCL, London, 7th-12th July, 2013.

Scholarship

Aug 2010 till June 2013: Recipient of Erasmus Mundus External Cooperation Window lot 13 Scholarship for doctoral studies (34 months).